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THE CHANGES OF *SHIGELLA SHIGAE* TOXIN TOXICITY INDUCED BY PHENOL

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SOME years ago we studied conductivity disturbances of the sciatic nerve after administration of *Shigella shigae* toxin (TSK) and Streptolysin O (SLO) in cats and rabbits (Raškova, Raška, Matějovská, Rybová, 1952). The local administration of both toxins caused identical characteristic conductivity changes, ending in complete block. After administration of phenol to the sciatic nerve analogous phenomena were seen.

In animals immunized previously with one of the two toxins no conductivity disturbances developed when the same toxin was locally administered in doses effective in control animals. Moreover, under these experimental conditions the other toxins and even phenol, in pretreated animals, were without the usual effect (Rašková, 1959; Rašková and Vaněček, 1957). In further experiments rabbits were pretreated with small doses of phenol and the same local adaptation phenomena were observed, but more phenol impulses were necessary than in the toxin experiments (Vaněček and Rašková, 1958). Thus pretreatment with phenol protected animals against conductivity disturbances which usually developed after local toxin administration to the sciatic nerve.

Now the question arose: would this hold true also for the general effects of the toxin as measured by LD_{50} changes? The results of an experiment designed in this way with *Sh. shigae* toxin are presented here.

METHODS AND MATERIAL

White mice of the Konárovice strain, kept at constant temperature and fed a standard diet were used for the experiment. At the beginning of the experiment the mice weighed 14–16 g. Twice a week 0·05 ml. of a 0·1 per cent solution of phenol was injected subcutaneously in various parts of the back. After 3, 6, 9, 12 and 15 doses of phenol the LD_{50} of *Sh. shigae* toxin was determined in the phenol treated group as well as in the control group taken from the same litters. The LD_{50} was established from 5 doses each on 5 animals. The whole experiment was repeated, so that the final LD_{50} was established from about 50

animals. (During the period of phenol treatment, some animals died accidentally so that the total number was a little below 50.) *Sh. shigae* toxin was prepared in a lyophylized form, the preparation of which was previously described (Raška, Rašková, Matějovská, Rybová, 1952). The toxin was administered intraperitoneally on the 3rd day and, in animals 12 times pretreated with phenol, also on the 14th, 28th and 42nd day after the last dose of phenol. In order to get information on the extent of the adaptive potency of phenol we performed one further experiment, for which the general conditions were the same, but instead of phenol, arsenic trioxide in a dosage of 40 μg. per mouse in 0·05 ml. saline was administered. Since we knew from the above experiments that changes could be expected only after frequent administration, doses of arsenic were given 10 or 14 times.

Groups were timed so that the final experimental comparison with the controls was performed on the same day, this was the 3rd day after the last arsenic administration. The LD_{50} of the *Sh. shigae* toxin was then determined in all groups.

RESULTS

All results from the phenol experiment were statistically analysed. All experimental and statistical data are in Table I. In all experiments the parameters $\bar{x}$ and $\bar{y}$, which define the relation between the percentage of deaths (in probit transformation) and the TSK dosage, were defined by successive approximation using the probit method. The deviation of observed and counted values did not exceed the limits necessary for homogeneous animal groups (Perry, 1950). From the data in Table I it is evident that the reactions of animals on increasing toxin dosage is characterised by a linear increase. Testing the variance about the dosage response line, there are at least 3 degrees of freedom and χ^2 for the 5 per cent limit is 7·8, all values in Table I are below this limit. Therefore we may characterise the laboratory animals used as a suitable group for this experiment.

Further the reproducibility of the experiments was analysed. This was possible as repeated control tests had been performed. The data are given in Table II. The individual LD_{50} were within the limits 0·100–0·126 and as the calculation proved, their variance is very small. Since $P = 0{\cdot}92$, this characterises the reproducibility of the experiments as very good. This judgment is supported by the evidence of parallelism of the regression lines, further support of the validity of the experiment.

It may be seen in Fig. 1A that there is an obvious effect of phenol pretreatment after 9 applications prior to the determination of the toxin LD_{50}. The protective effect was further increased after 12 doses, but after 15 phenol doses there was no further increase of protection. In this experiment, the LD_{50} was determined 3 days after the last phenol administration. In Fig. 1B the results of experiments where the TSK LD_{50} was determined 3, 14, 28 and 42 days after the 12th phenol injection are shown. These results show a gradual decline of the protective effect.

A further analysis of the available experimental data indicates that the protection obtained by phenol was not the only interesting phenomenon. It was possible to demonstrate, by an analysis of the data that the slope of the regression line of phenol pretreated animals was lower than that of the control group (Fig. 2), indicating that phenolized animals responded to the administration of toxin by a greater variation.

Arsenic trioxide in the dosage used (40 μg. per mouse, determined in preliminary experiments as a suitable tolerated dosage), has no influence on the

TABLE I

Experiment No.	Application	*Shigella shigae* toxin dosage						Linearity χ^2 test	LD_{50}	Regression coefficient
		$0{\cdot}7\times10^{-4}$	$0{\cdot}4\times10^{-4}$	$0{\cdot}23\times10^{-4}$	$0{\cdot}13\times10^{-4}$	$0{\cdot}7\times10^{-5}$	$0{\cdot}4\times10^{-5}$			
1	Phenol × 3	—	10/10	7/9	6/9	4/9	0/9	2·78	$0{\cdot}105\times10^{-4}$	0·84
1*a*	Control	—	10/10	6/8	6/9	3/10	0/9	1·96	0·114	0·91
2	Phenol × 6	—	9/9	8/9	5/9	4/9	0/10	1·82	0·105	0·93
2*a*	Control	—	10/10	8/10	6/10	3/10	0/10	0·83	0·116	0·92
3	Phenol × 9	—	7/8	5/9	3/8	2/9	0/9	0·92	0·174	0·69
3*a*	Control	—	10/10	6/8	6/9	3/10	0/9	1·96	0·114	0·91
4	Phenol × 12	5/8	3/8	2/10	1/8	0/9	—	0·50	0·515	0·58
4*a*	Control	9/9	10/10	9/9	5/9	2/8	—	0·57	0·111	1·23
5	Phenol × 12/14	4/7	4/9	2/10	0/7	0/7	—	0·77	0·515	0·67
5*a*	Control	8/8	9/9	7/7	5/9	3/8	—	1·29	0·100	0·99
6	Phenol × 12/28	5/6	6/8	4/7	2/6	1/8	—	0·36	0·212	0·58
6*a*	Control	10/10	10/10	7/10	6/10	2/10	—	1·72	0·126	0·84
7	Phenol × 12/42	—	7/9	3/7	4/9	1/8	0/8	1·76	0·203	0·63
7*a*	Control	—	9/9	6/8	3/7	3/8	0/8	0·35	0·122	0·85

TABLE II

Controls	LD_{50}	b
Mean	2·77	0·92
χ^2 test of reproducibility	1·29	1·16
Degrees of freedom	5	5
P	0·92	0·95

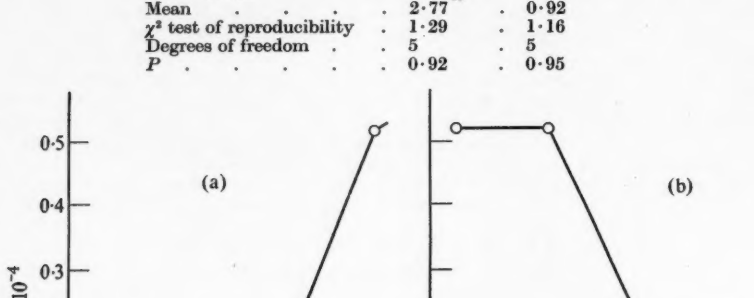

FIG. 1.—(*a*) The LD_{50} for mice of *Sh. shigae* toxin after increasing numbers of phenol doses. (*b*) The LD_{50} for mice of *Sh. shigae* toxin at various times after 12 doses of phenol.

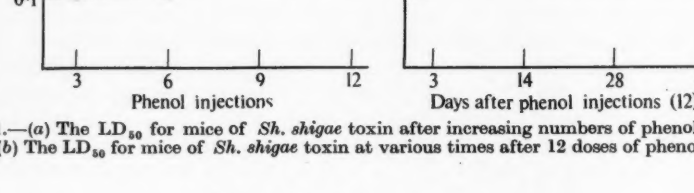

FIG. 2.—The dose response regression lines of control mice and mice pretreated with phenol for *Sh. shigae* toxin.

TSK LD_{50} after 10 or 14 doses. Since the data for experimental and control animals are almost identical, no statistical analysis has been performed.

TABLE III

Arsenic trioxide 40 μg. per mouse	*Shigella shigae* toxin dosage					
	$0·7\times10^{-4}$	$0·4\times10^{-4}$	$0·23\times10^{-4}$	$0·13\times10^{-4}$	$0·7\times10^{-5}$	$0·4\times10^{-5}$
Arsenic 10 times	..	9/9	7/9	5/9	3/9	0/9
Arsenic 14 times	10/10	10/10	8/10	6/10	3/10	..
Controls	10/10	10/10	7/10	6/10	3/10	0/10

DISCUSSION

In the introduction we have briefly summarised some of our experiments where repeated administration of one toxin leads to an adaptation to an antigenically unrelated toxin and also to phenol. However, this was studied on a local nervous reaction only. In other experiments an adaptation of animals to the effect of one toxin which also influences conditioning (*Salmonella typhi* endotoxin and Streptolysin O) was seen (Rašková and Vaněček, 1956). Non-specific adaptation in the field of bacterial toxins was long believed to be possible only between endotoxins (Thomas, 1954). The properdin system also is bound mainly to products of gram-negative bacteria (Landy and Pillemer, 1956), but within recent years evidence has shown that much wider mechanisms are involved. Dubos (1956) has shown that endotoxin makes mice more resistant not only to infections with gram-negative but also gram-positive bacteria, *i.e.* staphylococci or tubercle bacilli. This induced increased resistance lasted for several months. Our experiments, where animals were treated with one bacterial toxin to get adaptation to other toxins, could be compared with this work.

However, phenol is not an antigenic substance, yet it causes a definite increased resistance of mice to *Sh. shigae* toxin. In the future it will be interesting to see if this holds good for experimental infections. We thought at first that it might be possible to explain the phenol action by some tolerance mechanism, and therefore the experiments with arsenic trioxide were undertaken. This is a toxic drug to which the development of tolerance is well known and this tolerance may occur in mice after intraperitoneal injection (Norris and Elliot, 1945). However repeated injections of arsenic trioxide do not change the resistance of animals to *Sh. shigae* toxin, so for the moment it is hard to understand the mechanism of the protective effect achieved in our experiments with phenol. It is of interest that a comparatively large number of doses are necessary before increased resistance to the toxin appears, but this increased resistance to the toxin remains unchanged for a considerably shorter period than in Dubos' experiments. Furthermore the fact that repeated administration of phenol changed the reactivity of animals as compared with the controls had to be considered and therefore a further theoretical mathematical analysis was performed.

Let us suppose that for each animal there exists a certain toxin dosage for which a death probability equal to 1 is valid, and let us call it the individual lethal dose (x_k). This means that the probability of death for the animal receiving a dosage $x_k - \Delta x$ or smaller is nil and that it would only die after a dosage $x_k + \Delta x$ or more.

In the whole sample of animals the part

$$\frac{b}{\sqrt{2\pi}} \int_{x_k - \frac{\Delta x}{2}}^{x_k + \frac{\Delta x}{2}} e - \frac{b^2(x - \mathrm{LD}_{50})^2}{2}\, dx$$

will have the same individual lethal dosage. For easier understanding let us express the probability of death for these animals by the Poisson formula,

$$e^{-m}\,\frac{m^r}{r!}$$

where $m = 0$ and the null interval is in the limits $x_k - \Delta x/2$; $x_k + \Delta x/2$. There

is only one real solution and this is equal to 1. Now to this narrow homogeneous group phenol is applied. Suppose by this a positive interference into the reactivity of the organism, then some of the animals should die only after higher toxin doses than x_k. We can imagine that their distribution is again defined by the Poisson formula with a parameter >0, depending probably on the number of phenol impulses. In the general picture of distribution of the individual LD of phenolised animals this should show itself by a change of the LD_{50} and by a decrease of regression coefficient.

Now in the experiment both these theoretical suppositions have been really found. But there is still another point. Poisson frequency curves are very asymmetric for small values of the mean. A relation between the number of phenol impulses and such a parameter should be evident only after a greater number of impulses and this again was in fact so.

The described hypothesis, which presumes a discontinuous quantal relation between the toxin and the reaction of the living organism can from one side explain the results of our experiment.

This is in discrepancy with the view, that it would be possible to describe the phenol effect by a coefficient, indicating how much more toxin it would be necessary to take in comparison to controls to get the LD_{50} as is done in potency assays. Our analysis relates the effect of phenol to the parameter of probability that an animal which originally should die at its individual lethal dosage, dies only at a higher dose. This points to the complicated, non-specific mechanism of the phenol effect. Further work is necessary to elucidate this matter.

SUMMARY

The LD_{50} of the *Sh. shigae* toxin was compared in mice pretreated with small doses of phenol. From 9 phenol impulses on, mice developed a significantly increased resistance to the toxin. Arsenic trioxide under similar conditions was without effect. A detailed statistical analysis of the experimental material was performed. Measured by the slope of the regression line, pretreatment with phenol considerably changes the reactivity of the animals.

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ANTIBODIES AGAINST NUCLEAR AND CYTOPLASMIC CELL CONSTITUENTS IN SYSTEMIC LUPUS ERYTHEMATOSUS AND OTHER DISEASES

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GAJDUSEK (1957, 1958) showed that the sera of patients with systemic lupus erythematosus (SLE) and chronic hepatitis may contain complement fixing antibodies to human and animal tissues. Robbins, Holman, Deicher and Kunkel (1957) subsequently showed that sera of many patients with SLE reacted in the complement fixation test with nuclei and desoxyribose nucleic acid (DNA), of both human and animal origin.

This paper describes the results of complement fixation tests on sera from patients with SLE, chronic hepatitis, macroglobulinaemia and syphilis using human liver homogenates, rat liver nuclei and cytoplasm, calf thymus nuclei and calf thymus DNA as antigens.

MATERIALS AND METHODS

Antigens.—Human liver homogenate was prepared from autopsy material as 10 per cent suspension in 0·85 per cent saline (Gajdusek, 1958). Because of the difficulty in obtaining fresh human liver it was decided to make use of Gajdusek's observations that sera reacting against human tissue also reacted against animal tissue. For this reason rat liver was used in the study of the reactivity of the various cellular components.

Rat liver nuclei and calf thymus nuclei were prepared by the method of Mirsky and Pollister (1946). The rat liver was homogenized for 2 min. with a Teflon pestle with 9 vol. of cold 1 per cent citric acid. The calf thymus was homogenized in a Waring blendor with 19 vol. of cold 1 per cent citric acid. The citric acid lyses the mitochondria and inhibits the action of desoxyribonuclease. The nuclei were washed twice in 0·1 per cent citric acid and once in 0·25 M sucrose. Smears stained with methylene blue showed no unbroken cells or mitochondria.

Rat liver mitochondria and soluble cellular protein (supernatant) were prepared by a modification of the method of Schneider and Hogeboom (1950). The liver was homogenized with 9 vol. of cold 0·25 M sucrose with a Teflon pestle for 2 min. Nuclei and unbroken cells were removed by preliminary centrifugation at 500 *g* for 15 min. and the mitochondria isolated by centrifugation at 8000 *g* for 10 min. The mitochondria were washed in 0·25 M sucrose, kept overnight at −20° and rewashed twice in 0·25 M sucrose to remove soluble protein.

The microsomes were isolated at 40,000 *g* for 30 min. The residual fluid was then subjected to 2 centrifugations at 80,000 *g* for 40 min. to remove any remaining particulate matter. Care was taken to avoid contaminating the supernatant with the fatty layer that rose to the surface during high speed centrifugation. The final product remained clear on shaking and contained only soluble cellular protein.

Calf thymus DNA was prepared by the method of Signer and Schwander (1949).

Lipoid extract of whole rat liver was prepared by the methanol chloroform method of Rapport, Graf and Alonzo (1955).

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The cellular antigens were stored as a 20 per cent suspension in 0·25 M sucrose at −20°. The nuclear, mitochondrial and soluble cellular protein preparations were diluted with an equal volume of calcium magnesium saline (Gajdusek, 1958) before use. When the soluble cellular protein was highly anticomplementary it was used in a 5 per cent concentration. DNA was dissolved in physiological saline and used at a concentration of 100 μg./ml. The lipoid extract was used as a 10 per cent suspension.

Sera.—Sera from patients with SLE, lupoid hepatitis, primary biliary cirrhosis and macroglobulinaemia were obtained from the patients described by Mackay and Gajdusek (1958) and Mackay and Larkin (1958). Their system of code numbers has been followed in this paper; whenever possible the original sera employed by them were used. Two patients (R1 and R2) who presented with rheumatoid arthritis and a transiently positive LE cell test and 5 patients with syphilis and a positive Wassermann reaction were also studied. All the patients with SLE had given a positive LE cell test by the 2 hr. clot method of Magath and Winkle (1952) at some stage of their illness. Most of the patients were under treatment with cortisone and many were in remission at the time of bleeding.

Complement fixation test.—The sensitive method of Donnelley (1951) as modified by Gajdusek (1958) was employed. In order to overcome the anticomplementary activity of the antigens, complement was titrated in the presence of antigen and two 50 per cent haemolytic units of complement were used in the final titration. Antibody and antigen controls were included in each test.

Assuming a stoichiometric behaviour of the anticomplementary activity of the serum, even when the serum was not anticomplementary, an antibody titre of 1 in 4 might have been due to the additive effects of the anticomplementary activity of the serum and antigen; a titre of 1 in 8 was the lowest titre which might be valid. When the serum was anticomplementary, the lowest titre which could be attributed to a specific complement fixation reaction was two dilutions higher than the anticomplementary titre of the serum.

RESULTS

Complement fixing titres of abnormal sera were expressed as the reciprocal of the highest serum dilution giving 50 per cent haemolysis. The results of tests against human liver homogenate, rat liver nuclei, calf thymus nuclei, calf thymus DNA, rat liver mitochondria and rat liver soluble cellular protein antigens are presented in Tables II and III. The titres against the microsomal preparations were variable and are not recorded.

Purity of antigens.—On both immunological and preparative grounds the nuclear, mitochondrial and soluble cellular protein antigens were regarded as distinct. The separate nature of the mitochondrial and soluble cellular protein antigens was established by serial antigen dilution studies (Table I). When using sera which failed to react to a given antigen, serial antigen dilution titration set an upper limit to the contamination of the non-reactive antigen by the reactive antigens.

Systemic Lupus Erythematosus

Of the sera of 25 patients with SLE 4 reacted with rat liver nuclei, calf thymus nuclei and calf thymus DNA, rat liver mitochondria and rat liver soluble cellular protein, 5 reacted with rat liver mitochondria and rat liver soluble cellular protein, 6 reacted with rat liver mitochondria, while 10 failed to react with any of the antigens. It should be noted that reactivity against nuclear preparations was always accompanied by reactivity against DNA. In general, the reactivity against human liver homogenate paralleled that against rat liver cytoplasmic proteins.

TABLE I.—*Antigen Titrations. Minimum Strength of Antigen Preparations Reacting with Sera*

Code number of patient	Serum dilution	Rat liver nuclei (per cent)	Rat liver mitochondria (per cent)	Rat liver supernatant (per cent)
L1	1/10	N.R.	<0·078	2·5
L2	1/15	„	0·078	0·625
L3	1/50	0·312	0·312	—
L17	1/10	N.R.	5	0·625
	1/15	„	N.R.	0·313
L22	1/20	—	0·625	2·5
L30	1/20	—	<0·078	N.R.
L36	1/10	—	<0·078	0·625
R1	1/20	—	0·312	N.R.
HL 1*a*	1/10	—	0·625	1·25
HL 1*b*	1/15	—	N.R.	1·25
HB 1	1/15	—	0·625	0·625

N.R. indicates that there was no reaction with the antigen at full strength (10 per cent).
— indicates that no estimation was available.

TABLE II.—*Titres of Sera of Patients with Systemic Lupus Erythematosus against Human and Animal Antigens*

Code number of patient	Concurrent response to LE cell test	Calf thymus nuclei	Rat liver nuclei	Calf thymus D.N.A.	Rat liver mitochondria	Rat liver supernatant	Human liver
Nuclear and Cytoplasmic Reactors							
L3 (Jan. 1958)	Positive	16	8	16	8	—	—
L3 (May 1958)	„	64	64	16	64	16	—
L3 (June 1958)	„	0	0	0	0	0	4
L27	„	4	8	16	8	8	8
L28	„	0	16	16	8	4	16
L37	„	32	64	128	64	32	64
Mitochondrial and Soluble Cellular Protein Reactors							
L2	Negative	0	0	0	32	64	64
L17	„	0	0	0	4	32	—
L19	—	0	0	0	128	64	256
L22	Negative	0	0	0	32	8	32
L36	Positive	0	0	0	>128	>128	>128
Mitochondrial Reactors							
L1	Negative	0	0	0	256	0	32
L4	Positive	0	0	0	8	0	8
L6	„	0	0	0	8	0	0
L25	„	0	0	0	64	0	8
L30	„	0	0	0	128	0	128
R1	Negative	0	0	0	128	0	0
Non-Reactors							
L5	Negative	0	0	0	0	0	0
L7	„	0	0	0	0	0	0
L8	Positive	0	0	0	0	0	0
L11	Negative	0	0	0	0	0	0
L15	Positive	0	0	0	0	0	0
L16	Negative	0	0	0	0	0	0
L18	„	0	0	0	0	0	0
L21	„	0	0	0	0	0	0
L31	Positive	0	0	0	0	0	0
R2	„	0	0	0	0	0	0

— indicates that no estimation is available.

TABLE III.—*Titres of Sera of Patients with Lupoid Hepatitis, Primary Biliary Cirrhosis, Macroglobulinaemia and Syphilis against Human and Animal Antigens*

Code number of patient	Calf thymus nuclei	Rat liver nuclei	Rat liver mitochondria	Rat liver supernatant	Human liver
Lupoid Hepatitis					
HL1*a*	0	0	256	2,048	256
HL1*b*	—	—	16	1,024	—
HL3	0	0	0	0	—
HL5	0	0	0	0	—
HL6	0	0	0	16	0
Primary Biliary Cirrhosis					
HB1	0	0	1,024	1,024	128
Macroglobulinaemia					
MG3	0	0	>16,000	0	4,000
Syphilis					
S1	—	—	8	0	—
S2	—	—	64	64	—
S3	—	—	>128	>128	—
S4	—	—	64	0	—
S5	—	—	8	0	—

— indicates that no estimation is available.

Liver Disease

Of the 4 patients with lupoid hepatitis, one reacted with both soluble cellular protein and mitochondria, one reacted with soluble cellular protein only, while 2 failed to react with any of the antigens. However, none of these 4 patients had a concurrently positive LE cell test. The one patient with primary biliary cirrhosis (see Mackay, 1958) reacted with both mitochondria and soluble cellular protein in very high titres.

Macroglobulinaemia

One case of macroglobulinaemia reacted in extremely high titre to mitochondria but failed to react with the nuclei or soluble cellular protein.

Syphilis

All the 5 sera from syphilitic patients with a positive Wassermann reaction reacted with mitochondria and with lipoid derived from mitochondria. This is in keeping with the findings of Furth and Kabat (1941). Two sera also reacted with soluble cellular protein. Eleven of the high titre sera were tested against lipoid extract of rat liver and Eagle's heart antigen (Eagle, 1937). Only one of the patients with SLE (L30) reacted with these antigens.

Clinical Correlations in Patients with SLE

The 4 patients with positive reactions to nuclei and DNA also had a concurrently positive LE cell test. The converse was not true, for, of 9 other patients

with a concurrently positive LE cell test, 5 reacted only to human liver and rat cytoplasmic antigens, while 4 failed to react to any of the antigens.

The severity of the initial illness and the subsequent clinical course were graded by another observer (Mackay and Larkin, 1958). No definite correlation could be made between the presence and degree of renal, hepatic or haematological involvement and the clinical severity of the disease in individual patients on the one hand, and the corresponding titres and cell fraction specificities of the serum antibodies on the other.

DISCUSSION

These results demonstrate the occurrence in SLE of circulating complement fixing antibodies against both nuclear and cytoplasmic antigens. Circulating complement fixing antibodies against cytoplasmic antigens were also found in lupoid hepatitis, macroglobulinaemia, primary biliary cirrhosis and syphilis. The occurrence of the LE phenomenon in lupoid hepatitis suggests that antinuclear antibodies might have been found had more patients been studied.

All patients in the present series with positive nuclear and DNA reactions also showed a concurrently positive LE cell test. However, of 9 other patients with a concurrently positive LE cell test 5 reacted with cytoplasmic antigens only and 4 failed to react with any antigen. Thus the presence of the LE factor in the serum did not mean that circulating complement fixing antinuclear antibody would be detected.

The finding of complement fixing antibody against nuclei and DNA is in agreement with the work of Robbins, Holman, Deicher and Kunkel (1957). Friou (1958) and Friou, Finch and Detre (1958) used a fluorescent antibody technique capable of detecting complete and incomplete antibody, and demonstrated circulating antibody against nuclei in the sera of 27 out of 28 patients with active and quiescent SLE. Complement fixing antinuclear antibodies were detected in 4 out of 23 patients in the present series. As a sensitive method of complement fixation was used it is probable that the failure to demonstrate antibody against nuclei in many of the patients was due to an inability of the antinuclear antibody to bind complement.

The poor correlation between the serological and clinical findings in this series of patients with SLE may have been due either to the unimportance of circulating complement fixing antibodies or to certain special factors. *In vivo* absorption of antibody was a factor which could not be assessed. Only one specimen of serum was available from most patients and in many cases the natural history of the disease had been altered by treatment with cortisone. Nevertheless the poor correlation favours the view that the various complement fixing antibodies, particularly those against cytoplasmic components, are not themselves pathogenic in SLE but represent only one facet of altered immunological function.

Circulating complement fixing antibodies also seem unimportant in experimental auto-immune disease and in hypersensitivity of the delayed type. In experimental allergic encephalomyelitis there is no correlation between tissue damage and the titre of complement fixing antibodies (Lumsden, Kabat, Wolf and Bezer, 1950); Freund, Thompson and Lipton (1955) found no constant association between these factors in experimental allergic testicular damage. Similar observations have been made in experimental thyroiditis (Witebsky,

Rose, Terplan, Paine and Egan, 1957). In both experimental encephalomyelitis (Lipton and Freund, 1953) and in tuberculosis, skin hypersensitivity could be transferred passively by cells but not by serum.

When immunological damage cannot be attributed to a classical circulating antibody, then presumably either an incomplete circulating antibody is present, as seems likely in the case of the antinuclear and anti-DNA antibodies in SLE, or tissue damage is produced by immunologically competent cells without the mediation of soluble antibody. These possibilities are not mutually exclusive.

The lymphocyte and plasma cell infiltration seen in various organs including the liver (Mackay, Taft and Cowling, 1958) in SLE and related diseases may be due to a non-specific attraction of these cells to the sites of tissue damage and to the sites of antibody-antigen reactions. Alternatively a specific accumulation of immunologically competent cells may occur when antigens are present with which they can react. This hypothesis would explain the aggregation of mesenchymal cells and the occurrence of cellular damage in the absence of circulating antibody.

The auto-immune nature of the haematological changes in SLE rests on firm evidence (Dameshek, 1958). The present finding of complement fixing antibodies against both nuclear and cytoplasmic components of tissue cells in a proportion of cases of SLE supports the concept that a deep seated anomaly of immunological response is related to the pathogenesis of the disease. It also indicates that the presence of circulating antibody demonstrable by complement fixation is inconstant and does not seem to be an essential part of the disease picture.

SUMMARY

Previous observations of Robbins, Holman, Deicher and Kunkel (1957), on the occurrence of complement fixing antibodies against nuclei and desoxyribose nucleic acid in the sera of patients with systemic lupus erythematosus have been confirmed.

The sera of certain patients with systemic lupus erythematosus also reacted in the complement fixation test with soluble and insoluble cytoplasmic protein of rat liver.

Some patients with lupoid hepatitis, primary biliary cirrhosis, macroglobulinaema and syphilis reacted with cytoplasmic antigens, but none reacted with nuclei.

These findings support the auto-immune concept of the pathogenesis of SLE.

I am deeply grateful to Sir Macfarlane Burnet, Dr. Ian Wood and Dr. Ian Mackay for their encouragement and advice. I should also like to thank Miss Lois Larkin for detailed technical advice, and Mr. G. L. Ada for the preparation of calf thymus DNA.

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THE NATURE OF THE CONNECTIVE TISSUE DEFECT PRODUCED BY THE AMINONITRILES

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OBSERVATIONS on healing wounds (Enzinger and Warner, 1957), croton oil pouches (Mielke, Lalich and Angevine, 1957) and turpentine induced abscesses (Hurley, Storey and Ham, 1958) have shown that β-amino-propionitrile (BAPN) and related substances cause a delay in collagen maturation. To gain further information regarding this phenomenon the effects of BAPN and amino-acetonitrile (AAN) have been studied in two further experimental systems—the growth of granulation tissue into subcutaneously implanted polyvinyl sponges and the development of granulomata following the subcutaneous injection of carrageen.

MATERIALS AND METHODS

Sprague-Dawley and Wistar rats of either sex, both young (mean weight 50 g.) and adult (weight 150–200 g.) were used. The animals were fed a diet of Baristoc—a local proprietary food pellet—supplemented by fresh green vegetables. This diet and water were supplied *ad libitum*.

AAN was synthesized as its hydrosulphate by the method of Anslow and King (1941). BAPN was administered as its fumarate, obtained from Abbott Laboratories, Chicago. Each drug was injected subcutaneously daily.

Polyvinyl sponge (Prosthex, Raemer Chemical Co., London) pieces approximately 2 cm. square were implanted subcutaneously into the upper dorsal region by an aseptic technique.

Carrageen was prepared by boiling the dried seaweed, *Chondrus crispus*, with 100 times its weight of water for 30 min. and filtering twice through fine cotton gauze.

Material for histological examination was prepared and stained as previously described (Hurley *et al.*, 1958).

Material for estimation of hydroxyproline was weighed immediately after removal, cut into small pieces, defatted in 3 changes of acetone for a total period of 7 days and dried to a constant weight. A portion was hydrolyzed in a sealed tube with 4 N hydrochloric acid for 16 hr. at 110° (Levene and Gross, 1958). The hydroxyproline content was determined by the method of Neuman and Logan (1950) as modified by Martin and Axelrod (1953), using a " Unicam " spectrophotometer at a wave length of 558 mμ.

Because of the variation in different areas of the carrageen granulomata, 3 samples of approximately 50 mg. dry weight were taken from each granuloma.

RESULTS

Experiments with Polyvinyl Sponges

Sponges were implanted in 14 adult rats, half of which received 10 mg. of AAN per day. One injected and one control animal were sacrificed every week for 6 weeks. The remaining 2 rats received AAN for 5 weeks, injections were then discontinued, and the animals killed one week later—at the end of the 6th week.

Appearances in Control Animals

Macroscopic.—After one week the sponge was encapsulated by pink vascular tissue which required sharp dissection to separate the sponge without removing ingrowing material from its interstices. After 2 weeks the vascularity of the surrounding tissue had disappeared and over the succeeding 4 weeks little change in appearance was seen, but the mass of the sponge and its contents became progressively firmer.

Microscopic.—At the end of the first week ingrowth of tissue had filled most of the spaces in the sponge. This new tissue consisted of macrophages, occasional foreign body giant cells and numerous immature fibroblasts embedded in a ground substance containing fine argyrophil fibrils. The ground substance stained positively with Alcian blue, pale pink with PAS and green with the Picro Gomori stain. Numerous small blood vessels were present.

After 2 weeks (Fig. 1) macrophages had decreased in number, fibroblasts and ground substance were unchanged and numbers of coarse fibres with the staining reaction of collagen were present. Occasional fine argyrophil fibrils could still be seen.

From this time until observation ceased at the end of the 6th week (Fig. 3) collagen fibres appeared in increasing quantity. Fibroblasts decreased in number and altered in appearance to show small dark spindle nuclei and relatively little cytoplasm. Argyrophil fibrils disappeared, ground substance became less apparent and vascularity decreased. In addition, in specimens removed after 5 to 6 weeks, mature adipose tissue had replaced, in part, the developing fibrous tissue.

Appearances in Animals Receiving AAN

Macroscopic.—After one week there was no detectable difference from the control sponges. At all times thereafter, however, the sponges removed from animals receiving AAN showed greater vascularity than the corresponding control and even after 6 weeks the sponge from the AAN treated animal was significantly injected.

The sponges from the animals in which injections ceased one week before sacrifice were indistinguishable from the 6-week controls.

Microscopic.—After one week the reaction appeared identical with that seen in the control animal. Definite differences were seen at the end of the 2nd week (Fig. 2) and became progressively more obvious in later specimens. The macrophage reaction was not altered but the immature fibroblasts seen only in the first 2 weeks in the controls persisted until the 6th week (Fig. 4). Intercellular substance increased in amount, while retaining normal staining reactions. Argyrophil fibrils also persisted but very few collagen fibres appeared.

In the later stages fat was present as in the controls. The appearance of the sponges from the animals in which AAN administration ceased one week before sacrifice was indistinguishable from the 6-week control (Fig. 5).

Experiments with Carrageen

A volume of 1·5 ml. of 1 per cent carrageen extract and 1·5 ml. of air were injected subcutaneously into 24 adult rats, half of which received AAN 15 mg./day. Animals were sacrificed daily from the 3rd to 14th day.

Appearances in Control Animals

Macroscopic.—After injection the carrageen formed a firm oval swelling which was barely palpable by the following day. On the 4th day a semi-solid gelatinous mass, difficult to separate from the surrounding tissues, was found. From this time the size of the mass remained almost constant but became progressively firmer and more clearly defined. The early granulomata were pale pink and gradually lost colour with age.

Microscopic.—On the 3rd day the granuloma was composed of polymorphonuclear leucocytes and macrophages distributed in subcutaneous fat containing large blood vessels and scattered coarse collagen fibres. The macrophages contained abundant carrageen which stained deeply with Alcian blue, and a vivid red with PAS. By the 4th day a few immature fibroblasts had appeared and newly formed capillaries were first seen about the same time. Fine argyrophil fibrils appeared on the 6th day, were abundant by the 8th day, and joined in occasional areas to form fibres with the staining reactions of collagen. From the 9th to 14th day both the number of fibroblasts and the amount of collagen steadily increased (Fig. 7). The intercellular substance, staining positively with Alcian blue and pink with PAS was present only in small quantities. Carrageen-containing macrophages and occasional leucocytes were present at all stages.

Appearances in Animals Receiving AAN

Macroscopic.—For the first 9–10 days after carrageen injection the only differences detectable from the control granulomata were an increase in vascularity giving a pinkish colour and a somewhat softer consistence.

Over the next few days, however, a dramatic change occurred (Fig. 6). The mass decreased rapidly in size, to form a small soft area, occasionally with residual carrageen still visible in the centre, surrounded by a thin fibrous capsule. No coincident change in vascularity was seen.

During studies of several series of animals the decrease was first seen on either the 10th or 11th day and was of sudden appearance, the granuloma of the preceding day being of normal size.

Microscopic.—Over the first 6 days no difference from the controls was seen. By the 7th day argyrophil fibrils were present in approximately the same numbers as in the control, but the intercellular material appeared slightly increased.

By the 9th day a gross apparent increase in intercellular material was present and argyrophil fibrils were scattered through it (Fig. 8). The intercellular material showed its normal staining reactions. Fibroblasts were present in normal numbers but newly formed collagen fibres were not seen.

On the 11th day (after sudden decrease in size of the mass had occurred) the excess of intercellular substance was no longer seen and the dominant feature was masses of macrophages interspersed with coarse (preformed) collagen bundles and large blood vessels. By the 14th day very few fibroblasts were present, the mass consisting almost entirely of macrophages, densely packed together and still containing stainable carrageen.

Chemical

Two groups of animals were studied :

A volume of 1·5 ml. of 1 per cent solution of carrageen and 1·5 ml. of air were injected into 16 adult rats, half of which received, in addition, 15 mg./day

of AAN. Animals were sacrificed at intervals and the total wet and dry weights and the hydroxyproline content of their granulomata determined. The results obtained are contained in Table I.

TABLE I.—*Wet and Dry Weights, Hydroxyproline and Collagen Contents of Carrageen Granulomata from Control Rats and Rats Receiving AAN 15 mg./day*

Day	Granuloma weight in g. Wet	Granuloma weight in g. Dry	* Ratio dry/wet weight	Hydroxyproline mg./g. wet weight	† Total collagen in mg.	Comment
Control rats :						
2	2·465	0·181	0·073	0·59	10·8	
6	1·040	0·140	0·135	2·43	18·6	
8	2·840	0·315	0·111	2·26	47·3	An unusually large granuloma.
10	1·270	0·164	0·129	2·67	25·0	
11	1·225	0·231	0·188	4·38	39·4	
12	2·235	0·360	0·161	2·75	45·2	
13	2·370	0·434	0·183	4·66	81·3	
14	1·420	0·228	0·161	3·98	41·5	Granuloma smaller than usual.
Rats receiving AAN 15 mg./day :						
2	1·775	0·123	0·069	1·06	13·8	
6	1·005	0·121	0·120	2·12	15·7	
8	2·405	0·235	0·098	1·71	30·4	An unusually large granuloma.
10	0·555	0·102	0·184	3·68	15·0	
11	0·420	0·083	0·198	4·38	13·5	
12	1·105	0·191	0·173	4·65	37·6	" Shrinkage " less marked than usual. ? Very large granuloma initially.
13	0·530	0·114	0·216	5·40	21·0	
14	0·340	0·067	0·197	5·29	13·2	

* In both Tables I and II ratio dry/wet weight is given instead of water content as an unknown amount of fat was removed by acetone prior to dehydration.

† Total collagen $= \frac{\text{hydroxyproline}}{13{\cdot}6} \times 100 \times$ total wet weight.

Twenty-four adult rats received similar injections of carrageen and half were given 20 mg./day of BAPN. They were sacrificed at intervals as in the first group and the same determinations made. The results are contained in Table II.

Attempts to Define the Critical Period for the Macroscopic " Shrinkage " Effect

Two further groups of rats were studied :

A volume of 1·5 ml. of 1 per cent carrageen solution and 1·5 ml. of air were injected subcutaneously into 24 adult rats. The rats were divided into 8 equal groups.

One group received no further treatment. In the remaining 7, daily injections of 15 mg. of AAN were begun and administration discontinued at successive time intervals thereafter as indicated in Fig. 9.

All groups were sacrificed on the 14th day, the granulomata removed and weighed. All granulomata were of normal size except for the group receiving AAN for 11 days, in which definite " shrinkage " had occurred. The results are shown in Fig. 9.

TABLE II.—*Wet and Dry Weights, Hydroxyproline and Collagen Contents of Carrageen Granulomata in Control Rats and Rats Receiving BAPN 20 mg./day*

Day	Total granuloma weight (g.) Mean of 2 Wet	Dry	Ratio dry/wet weight	Hydroxyproline mg./g. wet weight	Total Collagen per granuloma (mg.)	Comment
Control rats :						
5	2·260	0·232	0·103	1·31	21·8	
7	2·040	0·248	0·122	1·74	26·1	
9	2·520	0·329	0·130	1·99	36·9	
11	2·745	0·311	0·113	1·52	30·7	
13	1·635	0·170	0·104	2·11	25·4	Granulomata smaller than usual.
15	2·243	0·358	0·160	2·99	49·3	
Rats receiving BAPN 20 mg/day :						
5	1·210	0·150	0·124	1·47	13·1	All granulomata a little smaller than usual, and than corresponding controls.
7	1·395	0·177	0·127	1·70	17·5	
9	1·542	0·196	0·127	1·76	19·9	
11	1·105	0·143	0·130	1·83	14·9	
13	0·735	0·131	0·178	2·98	16·1	
15	0·645	0·116	0·180	3·40	16·1	

EXPLANATION OF PLATES

FIGS. 1–5.—Each figure is a photomicrograph of a section of granulation tissue within an implanted polyvinyl sponge, stained with Van Gieson. × 180.

FIG. 1.—Control animal at 2 weeks. Note the large number of fibres present, mostly fine but some coarse and forming definite bundles.

FIG. 2.—Animal receiving AAN at 2 weeks. Note the presence of numerous fibroblasts and abundant intercellular substance in which are fine fibrils and large dilated blood vessels.

FIG. 3.—Control animal at 6 weeks. Bundles of coarse collagen fibres and areas of adipose tissue fill the interstices of the sponge, portions of which are present to the left and below.

FIG. 4.—Animal receiving AAN at 6 weeks. Some coarse collagen fibres are present, but most of the tissue closely resembles that in Fig. 2. Intercellular substance containing fine fibrils and dilated blood vessels is still prominent.

FIG. 5.—Animal killed one week after cessation of 5 weeks administration of AAN. Note the close resemblance to Fig. 3 ; coarse collagen fibres are present in large numbers and intercellular substance is scanty. Residual sponge is present on the right.

FIG. 6.—Each pair, A, B and C is a photograph, actual size, of two carrageen granulomata removed together with the overlying skin : to the left a granulomata from a control animal, to the right one from an animal receiving BAPN. A—treated animal (right) received 25 mg. BAPN per day. B and C—treated animal (right) received 20 mg. BAPN per day.

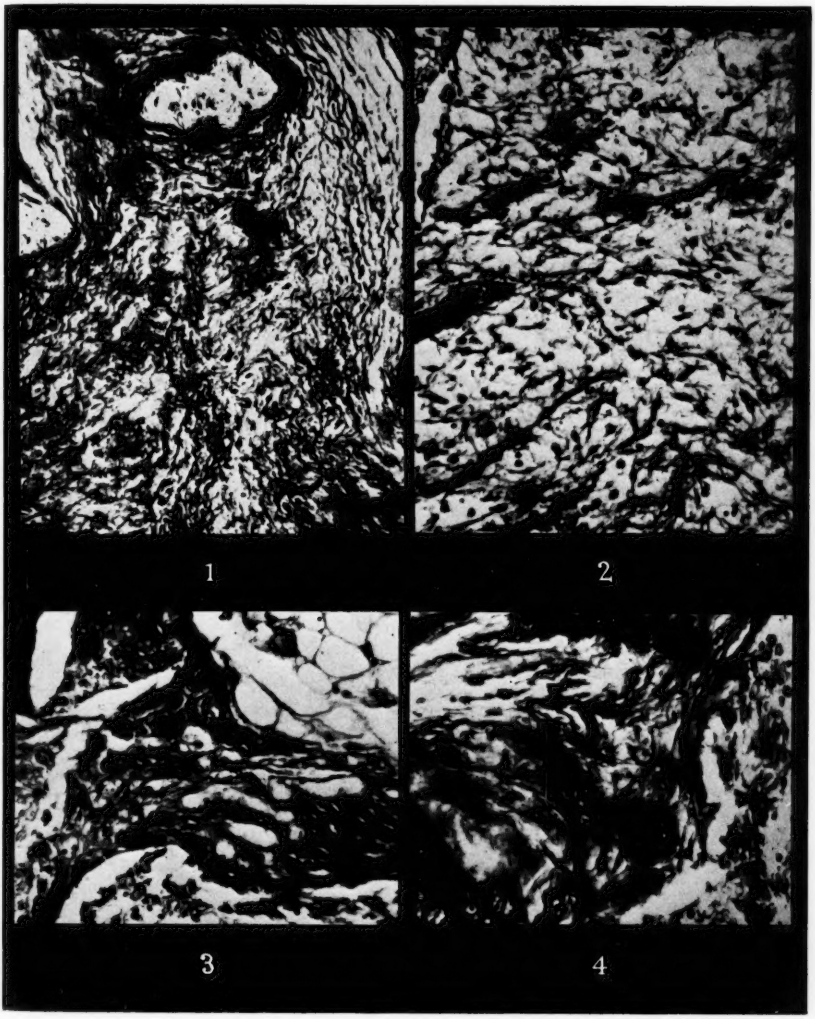
A—At 7 days. Note that well defined masses of similar size are present in both control and treated animals.

B—At 10 days. The granuloma from the control animal is similar to those seen in A, but more clearly defined : that in the treated animal is approximately half the size of the masses present at 7 days.

C—At 15 days. The appearance of the granuloma from the control is unchanged ; that from the treated animal is only a small fraction of the size of the granuloma from the 7-day treated animals seen in A.

FIG. 7.—Photomicrograph of a section of a carrageen granuloma from a control animal at 10 days. Note the large number of macrophages and fibroblasts and the moderate amount of intercellular material containing numerous fine fibrils. Stained with Van Gieson. × 180.

FIG. 8.—Photomicrograph of a section of a carrageen granuloma from an animal receiving AAN at 10 days. Many macrophages and fibroblasts are present embedded in abundant intercellular substance containing fine scattered fibrils. Dilated blood vessels are prominent. Stained with Van Gieson. × 180.



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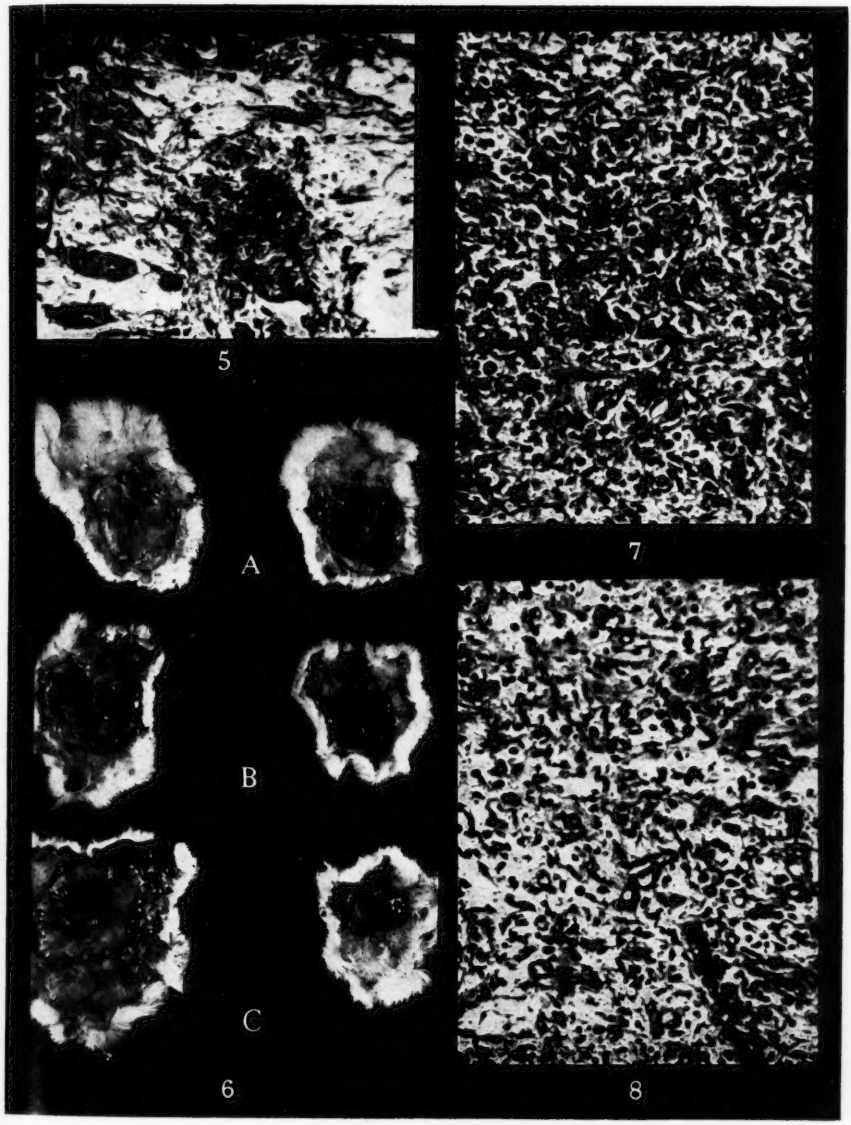

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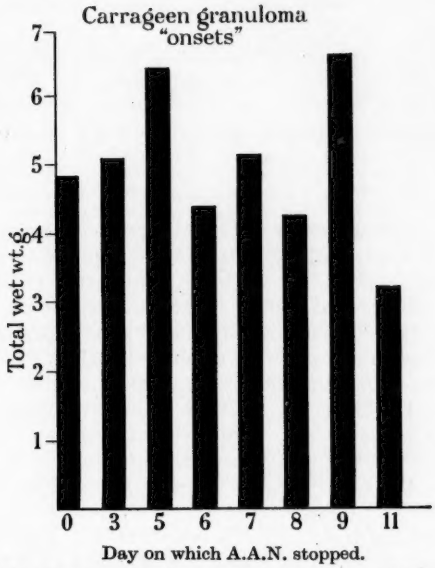

FIG. 9.—Wet weight of carrageen granulomata from animals in which AAN administration was started on the first day and stopped at various intervals. All animals killed on 14th day. Total weights are for groups of 3 animals in each case.

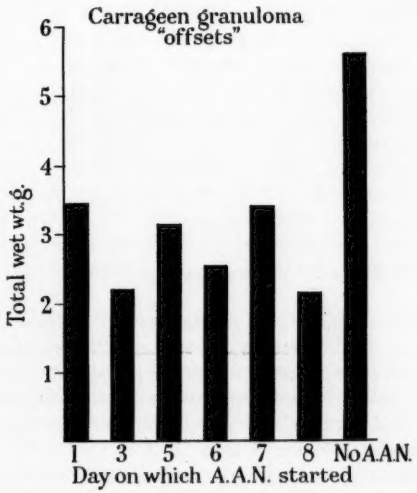

FIG. 10.—Wet weight of carrageen granulomata from animals in which commencement of AAN administration was delayed for various times. All animals killed on 14th day. Total weights are for groups of 3 animals in each case.

Twenty-four adult rats were similarly injected with carrageen and again divided into 8 groups.

Injections of AAN 15 mg./day were begun immediately in one group and at successive intervals thereafter in the other groups as shown in Fig. 10. All animals were sacrificed on the 14th day, the granulomata removed and weighed. All granulomata from animals receiving AAN showed " shrinkage ". The results are shown in Fig. 10.

DISCUSSION

The histological appearances in both polyvinyl sponge and carrageen granuloma preparations are essentially the same as the previously reported effects of aminonitriles on granulation tissue in both young and adult animals (Enzinger and Warner, 1957 ; Mielke *et al.*, 1957 ; Hurley *et al.*, 1958), namely : no change in the primary inflammatory response or in fibroblastic proliferation, an apparent increase in intercellular substance with unaltered staining properties and a delay in collagen formation. The increased vascularity noted in turpentine abscesses in lathyrous animals (Hurley *et al.*, 1958) is equally prominent in both polyvinyl sponge and carrageen preparations. The polyvinyl sponge experiments show that all these changes are reversible on cessation of aminonitrile administration.

If the percentage of hydroxyproline present be used as the index of collagen formation, Tables I and II demonstrate, at most, a slight retardation of collagen synthesis during administration of AAN and BAPN. Mielke *et al.* (1957) found that the collagen concentration in croton oil pouches of BAPN treated animals was slightly, but definitely, lower than that of the corresponding control pouches.

If, however, the total collagen present in the granuloma be used as the index of collagen formation, that is, the quantity of collagen formed in a given time in response to a uniform dose of carrageen, Tables I and II show that this figure increases two- to threefold between the 5th and 15th day in control animals, whilst in the animals receiving aminonitriles the total collagen present remains practically constant over the same period. This constant level must represent the preformed collagen present in the area of granuloma formation which can be clearly seen in the histological preparations, particularly those from the early specimens. These figures indicate that synthesis of new collagen practically ceases in the treated animals, whilst continuing actively in the controls.

If the results of Mielke *et al.* (1957) are rearranged as in Table III to show the total collagen present in the croton oil pouches of control and test animals, it is seen that here also the total collagen content remains constant from the 6th to 18th day in the test animals whilst a steady increase occurs in the controls over the same period.

In both the present carrageen experiments and in those of Mielke *et al.* (1957) occasional granulomata differ widely in total collagen content from the values anticipated by comparison with other specimens of similar duration. This variation is undoubtedly a reflection of the area over which the injection of carrageen or croton oil spread, and despite care to standardize technique as far as possible, represents an unavoidable source of error in the method used.

The experiment in which the beginning of AAN administration was delayed shows that commencement as late as the 9th day fails to inhibit the macroscopic " shrinkage " effect provided the drug be given during the period from the 10th to 15th day. If administration is begun on the first day and stopped at successive

TABLE III.—*Results of Mielke* et al. (1957) *Showing Wet Weights and Hydroxyproline Concentrations in Croton Oil Pouches from Normal and Test Rats fed BAPN, Rearranged to Demonstrate Total Collagen Content*

Day and rat type	Rat number	Wet weight of pouch in mg.	Weight of extracted protein in mg.	Hydroxyproline in extracted protein (per cent)	Total* collagen in extracted protein in mg.	Comment
6 controls	1	1810	138·6	2·70	27·5	
	2	1120	68·9	2·96	15·0	
	3	1130	106·2	2·97	23·2	
12 controls	4	1720	174·8	3·98	51·2	
	5	1700	159·4	3·80	44·7	
	6	1740	149·6	3·53	38·9	
18 controls	8	1410	124·2	4·26	39·0	
	9	1320	119·9	4·78	42·2	
	12	1340	97·1	4·16	29·8	
6 BAPN	13	540	44·6	1·89	6·2	
	14	650	33·9	1·65	4·1	
	15	1000	57·4	1·65	7·0	
12 BAPN	16	1450	122·5	3·66	33·0	Pouch unusually large
	17	670	37·8	2·48	6·9	
	18 & 24	810	62·5	2·94	13·5	
18 BAPN	25	580	42·5	1·88	5·9	
	29	530	26·8	3·52	6·9	
	33	810	35·9	3·52	9·3	

$$\text{* Total collagen} = \frac{100 \times \text{hydroxyproline per cent} \times \text{weight of extracted protein mg.}}{13{\cdot}6}$$

intervals thereafter, the animal can be given AAN for 9 days without subsequent shrinkage occurring provided no further drug is given. Thus the critical period for the drug's action is from the 10th to 15th day, and if AAN is given during this period, the "shrinkage" effect will occur whether or not the animal has previously been receiving the drug.

SUMMARY

Systemic administration of the aminonitriles, AAN and BAPN, to rats during the development of carrageen granulomata is associated with an apparently specific block in collagen maturation between the 10th and 15th day. At this time a sudden and extensive decrease in mass occurs, while control granulomata remain of constant size and histologically and chemically collagen formation continues actively.

The histological changes in granulation tissue in aminonitrile treated animals are similar in both carrageen granulomata and subcutaneously implanted polyvinyl sponges to those previously described in turpentine induced abscesses. In sponge preparations they are reversible on cessation of drug administration.

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OBSERVATIONS ON THE PATHOLOGY OF TOXOPLASMOSIS IN THE COTTON RAT

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INCREASING interest in the pathogenicity of *Toxoplasma gondii* has resulted in many species of laboratory animal being used as hosts for maintenance of the parasites, and the pathology of toxoplasmosis in animals has been described by many workers (*e.g.*, Wolf, Cowan and Paige, 1940; Frenkel, 1956). When the cotton rat (*Sigmodon hispidus*) was first used to produce large numbers of parasites for metabolic studies (Fulton and Spooner, 1957) it was found that after intraperitoneal injection of 2–3 million parasites the infection ran an acute course ending fatally on the third or fourth day.

More recently we have noticed some unusual pathological features of this disease in the cotton rat. Their nature and possible significance are the subject of this report.

MATERIALS AND METHODS

Young adult cotton rats weighing 120–150 g. were used. They were infected by intraperitoneal inoculation of 1 ml. of a suspension containing 2–3 million parasites of the RH strain of *T. gondii*, prepared by suitable dilution with phosphate buffer of the peritoneal exudate from an infected donor cotton rat. They were killed 3 or 4 days later, usually with chloroform. For comparative purposes some multimammate rats (*Mastomys coucha*) and mice, both of which are highly susceptible to infection, were similarly inoculated. The mice received a quarter of the rat dose.

Immediately after death peritoneal exudate, and in some cases also a pleural exudate, was removed by pipette. The volume of peritoneal fluid varied from 0–7 ml., the average being approximately 4 ml. The abdominal viscera were inspected for gross evidence of disease and pieces of the liver, kidney, spleen and pancreas, stomach, omental tissue and portions of the abdominal wall were removed for microscopical study. They were cut into slices not exceeding 5 mm. in thickness after 10–15 min. immersion in fixative. The pelvic viscera of males were removed *in toto* by block dissection. The fixatives used were Carnoy's fluid or, for the preservation of lipids, 10 per cent formal-calcium. Tissues were immersed in the former for not more than 1½ hr. and in the latter for at least 24 hr. Paraffin sections of 5 μ thickness were stained with haematoxylin and eosin or with Giemsa's stain. Frozen sections of the liver were cut at 10–15 μ, stained with Fettrot 7B (Ciba) or Sudan Black B and mounted in glycerin jelly. Three infected cotton rats were fixed by perfusion through the aorta with physiological saline followed by a solution of 10 per cent formalin containing 4 per cent acetic acid. Paraffin sections from representative levels of the central nervous system of these animals were stained with haematoxylin and eosin or with gallocyanin. From many of the Carnoy-fixed tissues additional paraffin sections were cut at 2–3 μ for examination by fluorescence microscopy after staining with the fluorochrome acridine orange at pH 2·7 for the histochemical differentiation of ribo- and deoxyribonucleic acids (Armstrong, 1956). Fluorescence was activated with the blue-violet emission of a 250 watt high pressure mercury vapour lamp.

For the specific investigation of the hepatic lesions found in the infected cotton rats histological observations were made on the liver in a further 103 young adult animals of both sexes, under the various experimental conditions specified below.

EXPERIMENTAL

Three days after inoculation with infectious peritoneal exudate, many of the cotton rats looked sick. Examination of males showed that in almost all, a white conical mass of firm, rubbery consistency covered the penis (Fig. 1). This mass varied in size, the largest being about 2·5 cm. long. Strong traction with forceps was needed to pull the mass free. It was then seen to be a cap-like structure with a thin stem extending into the urethra sometimes as far as the bladder. In infected animals of both sexes large areas of skin on the back and abdominal wall appeared to be swollen, and the subcutaneous tissue had a jelly-like appearance when freshly cut.

In the abdomen the peritoneal surfaces of the viscera were largely covered with adherent patches of white fibrinous exudate. It was most marked over the liver, spleen and stomach ; the omentum was thickened and sometimes adherent to other structures. The cut surface of the liver had a pale mottled appearance. A regular and conspicuous finding was enlargement of the pelvic viscera. The bladder was frequently distended and sometimes its wall was stretched so thin that the contents of the bladder could be seen. Usually it contained a mixture of urine and white material of cheese-like consistency ; the latter projected upwards from the urethral orifice into the lower part of the bladder as an irregular mass (Fig. 2). Urinary obstruction had clearly occurred in most of the infected animals. On either side of the bladder the white vesicular glands were conspicuously enlarged. The lobes of the ventral prostate gland, lying in front of the bladder, were also more prominent than in normal animals.

Histological Appearances

Pelvic viscera.—Paraffin sections stained by the routine methods invariably showed acute peritonitis and a severe inflammatory reaction in the interstitial connective tissues. Many parasites were also present. Most of the parasites were round or oval in outline and the crescentic form was only occasionally seen. Oedema, with capillary and lymphatic congestion was a feature of the connective tissues around the neck of the bladder and extended along interlobular planes into the vesicular, coagulating and prostate glands. The whole region was also infiltrated with large mononuclear cells arranged in clusters or strands ; these cells varied in shape but mostly had large oval nuclei and eosinophilic cytoplasm which was often vacuolated or foamy (Fig. 3). They will be referred to here as macrophages, but in places it was difficult to differentiate them clearly from fibroblasts of the surrounding tissue. Mast cells were present among the clusters of macrophages. The peritoneal mesothelium was heavily parasitized, as were many of the macrophages in the underlying tissues. In the prostate gland parasites were most numerous close to the peritoneal surfaces, but in many places had evidently penetrated deeply by way of the connective tissue planes. They were both intracellular and extracellular in position (Fig. 4). Frequently the cytoplasm of the affected cells was virtually replaced by parasites (Fig. 5). Mitotic figures were occasionally seen even in the heavily parasitized macrophages. The secretory epithelium, however, was conspicuously free from parasitic invasion even where the adjacent supporting tissues were heavily infiltrated. Masses of free parasites occurred here and there in the interstitial tissue associated with localized areas of fibrinoid necrosis. Occasionally, nearby areas of the

secretory epithelium had become involved in the necrotic process, often with formation of microscopic abscesses showing bacterial contamination. The vesicular and coagulating glands showed similar but less extensive parasitic infiltration; both were markedly distended with secretion. The testis and epididymis were not involved and looked normal in the animals studied.

The bladder wall had been invaded by parasites from the peritoneal surface, and they were often to be found in the muscle coat. The white material contained within the bladder consisted largely of granular, eosinophilic material mixed with hyaline strands and occasional groups of spermatozoa. Sections through the external penile cap showed similar characters, and it was continuous through the urethra with the bladder contents. It was evidently an abnormal seminal secretion derived mainly from the prostate and vesicular glands.

Liver.—A thin layer of fibrinous exudate covered the peritoneal surfaces, and parasites could usually be found embedded within it; intracellular forms were limited to occasional mesothelial cells, macrophages and leucocytes in the superficial zone of peritonitis. There was no sign of parasitic infiltration of the liver substance; minute focal necroses were a rare finding, and massive necrosis of the liver did not occur. However, a pattern of periportal vacuolation of the hepatic parenchyma was always present. In frozen sections stained for lipids, this was seen to be due to accummulation of intracellular fat. Its distribution and amount was much the same in sections stained with Sudan Black B and Fettrot 7B suggesting a high proportion of neutral fats. The liver of the cotton rat in toxoplasmosis was quite different from that of *Mastomys* or mice infected with the same strain of parasites. In the 2 latter species intraperitoneal infection is followed by extensive parasitic invasion of the liver; intracellular forms are found both in the Küpffer cells of the sinusoids and in the liver cells themselves (Fig. 8 and 9). Fatty infiltration is not found but widespread focal necroses occur in the vicinity of the parasites, often becoming confluent or massive. Further investigation of the zonal fatty infiltration seen only in the cotton rats is reported below.

Other organs.—Sections of omental tissue revealed an acute inflammatory reaction essentially the same as that described in the mouse omentum by Cross and Anigstein (1949) with considerable numbers of free and intracellular parasites. The spleen was congested and heavily infiltrated with polymorphonuclear leucocytes. Intrasplenic parasites were rare, but occurred in the cytoplasm of cells lining the sinusoids. A variable amount of superficial necrosis was always present on the peritoneal surfaces of the spleen, pancreas, stomach and kidney but no other specific lesions were seen. Renal damage from urinary obstruction was not apparent, presumably because of its short duration. Sections of the brain, spinal cord and meninges of 3 cotton rats fixed by perfusion revealed no parasites in the central nervous system, which appeared to be quite normal.

Skin.—Sections revealed that swelling of the skin was attributable largely to an oedematous condition of the dermis and subcutaneous connective tissue. Over large areas there was no visible cellular reaction, but here and there groups of vacuolated macrophages were found similar to those described in the connective tissues of the prostate. Mast cells were also prominent. Free and intracellular toxoplasms were also found, they were most numerous in and around the subcutaneous muscle sheet, but sometimes were found just beneath the epidermis in close proximity to hair follicles and associated glands.

Fluorescence microscopy

The acridine orange fluorescence technique proved to be a useful supplementary method for the study of *T. gondii* in sections of infected tissues. Sectioned parasites stood out well on account of a distinctive fluorescence which contrasted with that of the connective tissue elements of the host (Fig. 6). At low and intermediate magnifications the parasites seemed to be emitting an orange-red fluorescence, but under oil immersion each was seen to consist of a red fluorescent peripheral zone and a small, bright yellow central body. This finding is in accordance with the view that within each parasite a central " nuclear " structure containing deoxyribonucleic acid is surrounded by cytoplasmic material with an appreciable ribonucleic acid content. It was found that in some tissues containing relatively few parasites, such as the skin, it was possible to locate them more easily with the fluorescence microscope than in Giemsa or haematoxylin stained sections.

Fatty infiltration of the liver

There could be little doubt that periportal fatty infiltration was a consequence of infection with *T. gondii*. The livers of 10 normal cotton rats from the same stock showed no sign of fatty accumulation. The routine use of chloroform for killing infected animals was not responsible since 4 infected animals killed with coal gas or by a blow on the head all showed the typical pattern of fatty change. A series of experiments was therefore devised to see whether a comparable change could be produced other than by active infection with *T. gondii*.

Twelve cotton rats were injected intraperitoneally with 1 ml. of a suspension of toxoplasms which had been freed from other cells (Fulton and Spooner, 1957) and then broken by ultrasonic treatment (Donald and Isaacs, 1954) and finally frozen (−79°) and thawed twice to ensure the death of any viable parasites. The animals were killed with chloroform at intervals up to 5 days after inoculation. No signs of illness were noted, and the improbability of accidental infection with living parasites was shown by keeping similarly inoculated animals under observation for periods up to 6 weeks as controls. Periportal fatty infiltration was

EXPLANATION OF PLATES

FIG. 1.—A male cotton rat 3 days after intraperitoneal infection with *T. gondii*, showing a well formed white mass attached to the penis.

FIG. 2.—Dissection of the distended bladder of a male cotton rat which had been infected for 3 days. A plug of white material rises from the urethra into the bladder. ×3.

FIG. 3.—Section of ventral prostate gland from an infected cotton rat, showing a group of vacuolated macrophages in the sub-epithelial connective tissue. At the centre are two mast cells. Haematoxylin and eosin. ×470.

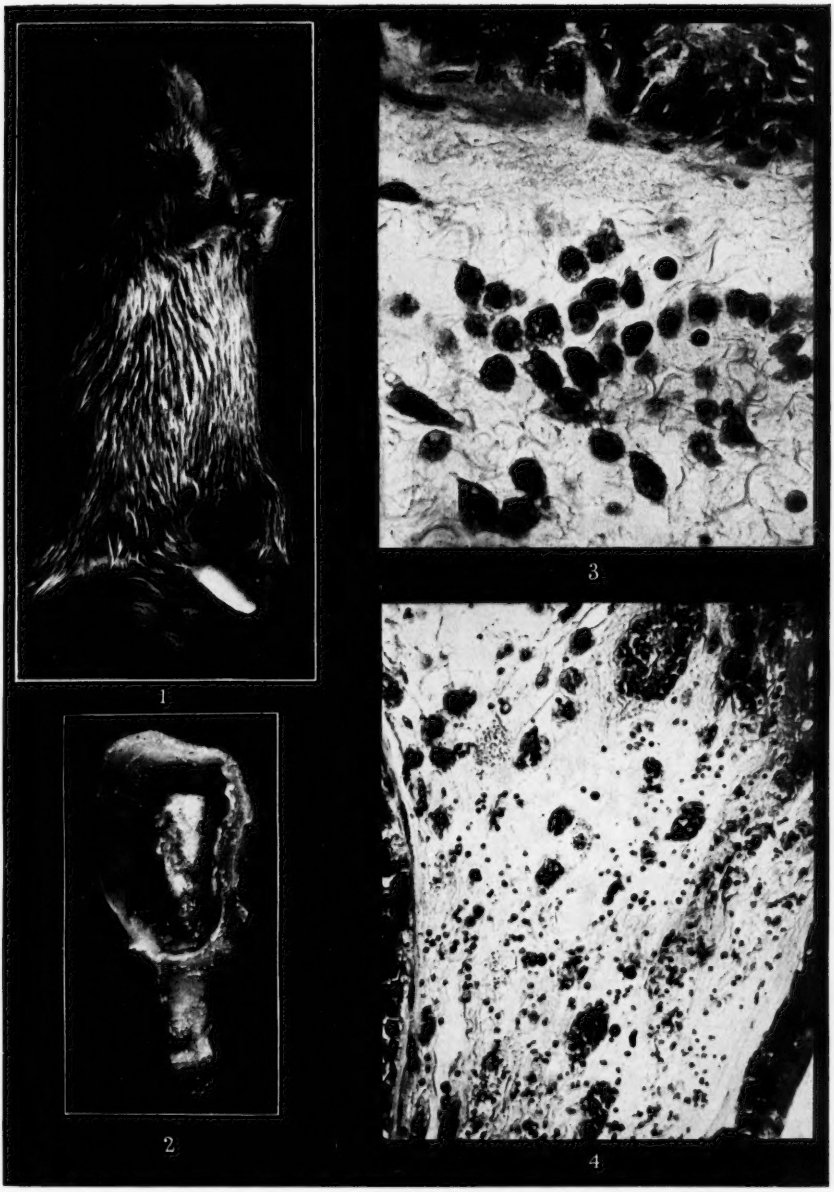
FIG. 4.—Section of prostate gland from an infected cotton rat. The interstitial connective tissue is extensively infiltrated with parasites. Haematoxylin and eosin. ×385.

FIG. 5.—Section showing a macrophage, heavily loaded with parasites, in the prostate of a cotton rat. The cell nucleus is fragmenting. Haematoxylin and eosin. ×1335.

FIG. 6.—Section of the vesicular gland of an infected cotton rat, as seen with the fluorescence microscope, after treatment with acridine orange at pH 2·7. Many brightly fluorescing parasites are shown here in the sub-epithelial tissue. ×450.

FIG. 7.—Frozen section of cotton rat liver, 2 days after inoculation with killed parasites. Note the periportal fatty infiltration. Fettrot 7B. ×33.

FIG. 8 and 9.—Sections of liver of a multimammate rat (*Mastomys*) 3 days after infection with *T. gondii*, showing parasitic invasion of hepatic parenchyma. In Fig. 8 is an early stage (rosette form). Giemsa. ×1250. Fig. 9 shows a later stage. Giemsa. ×835.



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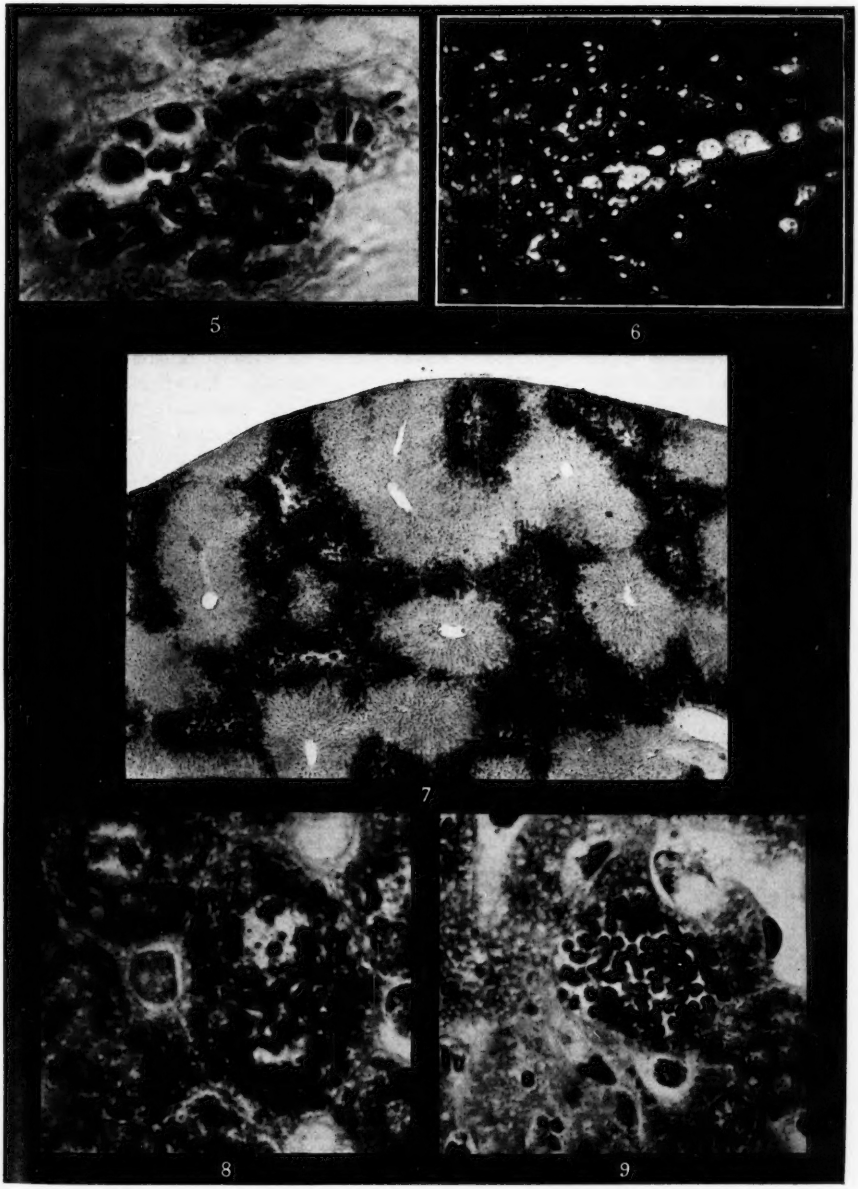

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found in various degrees in all animals of this group. In those killed after 24 hr. it was slight to moderate ; but on the second day, there was considerable fat present and the liver had the same appearance as in animals dying of toxoplasmosis (Fig. 7). In one animal, distribution of fat was not zonal but generalized, and exceeded in amount that found in the infected cotton rats. After 4 days rather less periportal fat was found, and in animals killed on the fifth day only occasional large droplets of stainable fat remained.

Twenty-one cotton rats were given intraperitoneal injections of 1 ml. of the supernatant from centrifuged peritoneal exudate of toxoplasma-infected donors ; the inoculum was also twice frozen (−79°) and thawed before use. Periportal fatty infiltration of the liver was found in these animals just as in the previous group, the accumulation of fat being maximal in those animals killed on the second and third days after inoculation. Very little was found in those killed on the fifth day. Some of the same inoculum was given to 8 cotton rats after being stored for 10 weeks at −2°. This time only slight to moderate fatty infiltration was found. From tests on a further 8 animals it was deduced that dialysis of this inoculum against distilled water for 2 days before use did not in any way alter its capacity to induce fatty infiltration.

Ten cotton rats were inoculated with Seitz-filtered products of *in vitro* metabolism of *T. gondii*, kept for 2 hr. in a suitable medium (Fulton and Spooner, 1957). A small amount of periportal fat was found in the animals killed 24 hr. after inoculation but the remainder up to 4 days after inoculation showed none.

For comparison with the above, 16 cotton rats were inoculated with materials unrelated to *T. gondii*. Half of them received an exudate produced in donor animals by intraperitoneal injection of turpentine (0·1–0·2 ml. given 3 days earlier); the remainder were injected with a suspension made from *Trypanosoma rhodesiense* which had been washed and then broken up by ultrasonics. A very small quantity of stainable periportal fat was found in the liver in some of these animals, notably after 2 days. It was variable in amount and the reaction never approached in severity that regularly found in cotton rats with toxoplasmosis or after inoculation with preparations derived from *T. gondii*.

Finally, 9 normal cotton rats were allowed water but no solid food for 24 hr. before death and 9 others were similarly prepared for 48 hr. In 8 out of the first 9, a considerable degree of periportal fatty infiltration had developed, comparable with that seen in infected animals. All 9 of the rats deprived of food for 48 hr. showed severe fatty change in the liver ; in some it reached massive proportions and was not limited to the periportal areas.

DISCUSSION

The appearance of an unusual secretion on the penis of male cotton rats infected with *T. gondii* led us to investigate further the pathology of this disease in several hundred specimens of the new laboratory host. The causal relationship between this infection and the secretion became apparent. Inspection of normal male cotton rats in the large breeding stock revealed no sign of an external secretion, but a similar condition was observed in one adult male which had been isolated for some weeks. Infection of immature males with *T. gondii* has never produced this condition in our experience. Similarly, in large numbers of cotton rats infected for several months with *Leishmania donovani* the condition has not

been observed by us. It is concluded that the development of the secretion is due to continuous irritation of the pelvic accessory glands of fully developed male animals, as a result of the acute inflammatory reaction which follows infection with *T. gondii*.

Mice have been widely used as susceptible hosts in the study of toxoplasmosis, but so far as we are aware the occurrence of a penile secretion has not been described. However, examination of 60 adult male mice infected 3 or 4 days previously with *T. gondii* showed that the condition was in fact present in 20 per cent of the group. It was never observed in control mice and so far has not been seen in the limited number of infected multimammate rats at our disposal. It is therefore clear that the secretion forms in response to a specific stimulus and is not wholly dependent on the host species.

The state of the liver in toxoplasma-infected cotton rats is interesting in two respects. First, parasitic invasion does not occur in spite of the peritoneal exudate being exceptionally rich in parasites; secondly, a marked degree of zonal fatty infiltration is a constant feature. These findings are contrary to those in mice and multimammate rats infected with the same strain of *T. gondii*. Variation in the tissue reactions of different hosts when infected with a given strain of *T. gondii* has been indicated by Frenkel (1953), but the regular occurrence of periportal fatty infiltration seems not to have been described previously in experimental toxoplasmosis. The precise significance of the fat accumulation is uncertain. It is not found in normal cotton rats, but can be produced by inoculation either with disrupted parasites or with the supernatant of peritoneal exudate from which all parasites have been removed. Such animals remain well and the fatty changes in the liver are transient, being maximal some 2 days after inoculation. The specificity of this reaction is evident from the failure of other kinds of peritoneal exudate to produce it. In this connection it may be recalled that infection of cotton rats with *Leishmania donovani* by the intraperitoneal route did not induce fatty infiltration of the liver (Fulton and Niven, 1951), although parasitic invasion of the liver did occur in that case. From the experiments on uninfected animals in which food was withheld for up to 48 hr. before death it is clear that the cotton rat has a definite predisposition to fatty infiltration under conditions of restricted diet. However, nutritional deficiency is unlikely to be the whole explanation of the picture in toxoplasmosis since during the course of the infection there is no serious impairment of food intake. Moreover, animals inoculated with killed parasites (or with supernatant of centrifuged exudate) showed no signs of illness and behaved as normal animals. It is concluded that some substance, or substances, produced during growth and multiplication of *T. gondii* may be responsible for initiating periportal fatty infiltration. Cook and Jacobs (1958) found no evidence of a cytotoxic effect of *T. gondii* in tissue culture studies. The peritoneal exudate of infected animals was however shown by Weinman and Klatchko (1950) to be rapidly lethal for mice when injected intravenously. The peritoneal exudate from cotton rats is similarly toxic for mice but the same factor cannot be responsible for fatty changes in the cotton rat liver, since it was found that the suspension of purified parasites, broken up by ultrasonic treatment, was not harmful to mice when injected into the tail vein.

The oedematous swelling of skin and subcutaneous tissue and the presence of parasites around hair follicles suggest the possibility of spread of infection

by contact in an animal colony. An extended study of this question has yet to be made. It is known however, that the disease is not transmitted in this way to clean mice kept in contact with infected animals for many months (Lainson, personal communication).

The cause of the invariable death of cotton rats on the third or fourth day of infection remains obscure. Extensive necrosis of the liver could be an important factor contributing to the death of infected mice and multimammate rats, but this is clearly not so in the cotton rat. Urinary obstruction, although present to some extent in almost all of the male cotton rats, is unlikely to have caused significant renal insufficiency in the time available. Moreover, no difference was seen between the survival times of male and female animals. The presence within the peritoneal and pleural cavities of an exudate which is highly toxic for mice might itself be regarded as an adequate reason for the fatal outcome.

SUMMARY

An unusual penile secretion, found in adult male cotton rats after intraperitoneal infection with *T. gondii*, is accompanied by urethral obstruction and retention of urine. It appears to result from parasitic invasion and acute inflammation of the pelvic organs.

Hepatic lesions in cotton rats with toxoplasmosis are unlike those observed in other rodents similarly infected. The significance of fatty infiltration of the liver was investigated and is discussed.

Oedema and parasitic infiltration of the skin is a notable feature in many infected cotton rats, suggesting a possibility of cross infection by contact.

In tissue sections stained with acridine orange it is frequently possible by fluorescence microscopy to locate the parasites more easily than by ordinary staining methods.

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THE PITUITARY REMNANT AFTER PARTIAL HYPOPHYSECTOMY IN THE RAT

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THE question as to whether the anterior lobe of the pituitary regenerates after partial destruction has excited interest recently, following reports of necrosis of a major proportion of the pars distalis resulting from stalk section in man (Russell, 1956; Daniel, Prichard and Schurr, 1958), in the rat (Barrnett and Greep, 1951; Daniel and Prichard, 1956) and in the sheep and goat (Daniel and Prichard, 1957, 1958).

It has been claimed by some authors that the anterior pituitary possesses powers of regeneration, if the preceding destruction has not been too extensive (Cameron, 1952) and this is based on the reported incidence of mitoses in the normal gland (Majima, 1926; Weis, 1935; Baer, 1940), after injury and after endocrine manipulations (Wolfe, 1935; Pomerat, 1941). This view is not in agreement with observations made on the regenerative power of fragments of pars anterior following attempts at total hypophysectomy in small animals. Thus Riddle, Smith and Morgan (1935) asserted that regeneration was poor in pigeons following "failed total hypophysectomy"; Richter and Wislocki (1930) reported that, 88 days after attempts at pituitary extirpation in the rat, the residual fragments, consisting in most cases of pars anterior, amounted to less than whole pituitary glands, and Smith (1932) found that fragments which survived "failed total hypophysectomy" in the rat and those which were intentionally spared showed no increase in mass, although he was not able to determine what proportion of the gland had been removed at the time of operation. On the other hand, Reiss, Epstein and Gothe (1937) described a remarkably rapid and complete compensatory hypertrophy in the fragments of pars anterior after failed total and intentional partial hypophysectomy in the rat. Crooke (1938) referred to a cellular hypertrophy in fragments accidentally left in the sella. Daniel and Prichard (1958) considered that regeneration may have occurred in the anterior pituitary of goats which were allowed to survive for $1\frac{1}{2}$ to 4 weeks after stalk section.

Assessment of regeneration is known to be difficult and conclusions may be erroneous when due regard is not paid to various conditions such as the proportion resected, integrity of the blood supply and to the control of fluctuations in size of the organ due to processes other than regeneration (Harkness, 1957). These precautions do not appear to have been observed in the reported work on the pituitary.

In the experiments to be described we attempted to develop a standard procedure for partial hypophysectomy leaving a known proportion of pars

anterior in its usual relationship with the pars nervosa and hypothalamus, and maintaining at the same time an intact blood supply. We then observed the remnant, attempting to analyse alterations in weight, cell population and function at various times after partial resection. In order to test if functional adaptation had taken place in the pituitary remnant, we compared goitres induced by thiouracil immediately after partial hypophysectomy with those induced one month later. It is generally accepted that thiouracil interferes with the production of thyroxine and that the resultant low blood thyroxine level is followed by an increased secretion of thyrotrophic hormone by the anterior pituitary. Thyroid hyperplasia is stimulated in this way and both total gland weight and follicular cell height are increased (Astwood, Sullivan, Bissell and Tyslowitz, 1943 ; Mackenzie and Mackenzie, 1943 ; Astwood, 1949).

MATERIAL AND METHODS

Rats.—Male and female albino rats, mostly 120–200 g. weight at the time of operation and bred in our laboratory, were fed on " Research " rat cubes. At birth all litters were reduced to 8, the babies being weaned after 3 weeks.

Operative procedures

Sagittal partial hypophysectomy.—The technique followed, with some modifications, that described for total hypophysectomy by Smith (1930) and Thompson (1932). A drawn-out polythene catheter was used for per-oral laryngeal intubation. The anterior lobe of the pituitary was incised sagittally in the midline with a No. 12 curved Swann-Morton scalpel blade. The right half of the gland was then sucked out with a glass cannula in which a trap had been blown so that the fragment could be recovered, weighed and fixed for subsequent sectioning. The remnant was then examined *in situ* under dissecting microscopic vision ($\times$ 12–15), (Fig. 1 and 2).

Coronal partial hypophysectomy.—In some cases only the caudal part of the pars anterior was removed, leaving the portal vessels of both sides fanning out over the remnant. The pituitary was incised transversely with sharply pointed de Wecker's ophthalmic scissors. The caudal part of the gland was either lifted or sucked out. This procedure was more difficult than the sagittal operation and the resected tissue was usually smaller. A greater resection would have resulted in damage to the underlying pars nervosa (Fig. 3).

Post-operatively the rats were placed overnight in a chamber maintained at 32·5° and returned to the stock animal room the following day.

Ether inhalation was used for killing rats.

Weighing

Pituitary glands, resected portions and remnants were weighed in air-tight glass tubes on an automatic aperiodic balance and then immediately fixed in Helly's fluid. The tissues were usually weighed a second time after 2 hr. fixation. When ratios of pars anterior to pars nervosa and pars intermedia were calculated, the lobes were separated under the dissecting microscope after 2 hr. fixation. The fresh and fixed weights differed only slightly in this series.

Thyroid glands were fixed attached to the trachea for 8 hr. in Helly's fluid and after 12 hr. washing were dissected free under microscopic vision and then weighed on a 1 g. torsion balance. Adrenals were weighed fresh on a 1 g. torsion balance.

Methylthiouracil

The 4-methyl-2-thiouracil compound was administered as a saturated solution, prepared by suspending 1 g. in 1 litre of drinking water. This was given to the rats for a period of 14 days.

Microscopy

Tissues were embedded in paraffin and most were cut at 5 μ. Single sections of the thyroids and serial sections of the resected and residual pituitary tissues were stained with Periodic Acid Schiff and Orange G (Pearse, 1949).

Differential cell counts were made on alternate fields in every 20th section under oil immersion using a square ocular, the field measuring 2500 sq. μ. Approximately 4000–8000 cells per pituitary gland were counted.

Serial coronal sections of the hypothalamic regions in selected cases were cut at 7 μ beginning immediately rostral to the optic chiasma and ending caudal to the mamillary bodies. Every 7th section was mounted and stained with Haematoxylin and Eosin.

Thyroid follicle cells were measured according to the technique of Logothetopoulos and Doniach (1955), in adjacent fields over the whole section at a magnification of $\times 900$. A linear ocular micrometer was used which could be rotated to lie directly over the long diameter of follicle cells: 100 cells were measured in each gland.

Cases were discarded from the main data if at the original operation the resected part was not recovered in 1 or 2 pieces. When the tissue fragmented (as happened several times in the early stages of the work) we were not certain that the small fragments were completely recovered for weighing. Further experiments were rejected if there was microscopical evidence of infection with substantial polymorphonuclear infiltration, or if on microscopy it was evident that the pituitary had been weighed with a tag of fibrin attached, or if the animal had developed severe respiratory infection.

Experimental procedure

Group I.—In order to determine whether the pituitary bore a constant relationship with body weight, 60 male and 48 virgin female rats of varying ages were killed and the whole pituitary immediately weighed.

Group II.—Sagittal partial hypophysectomy was performed in 10 male and 1 female rats which were killed immediately and the pituitary remnants were then weighed. The actual remnant weights were compared with the estimated weights of the remnants, as derived from the regression equations.

Group III.—Sagittal partial hypophysectomy, removing approximately one-third of the estimated anterior lobe was performed in 35 male and 27 female rats, which were killed at various times after the operations as follows: Of the males, 9 were killed after 1 week; 13 after 3 weeks; 5 after 5 weeks; and 8 after 9 weeks. The females were killed in three groups: 9 after 2 weeks; 10 after 6 weeks; and 8 after 8 weeks.

Group IV.—Mock operations, which included drilling of the basisphenoid and exposure of the pituitary, were carried out in 25 male and 18 female control rats. The males were killed in groups of 5 after 1 week; 7 after 3 weeks; 5 after 5 weeks; and 8 after 9 weeks. The females were killed in groups of 9 after 2 weeks; 7 after 6 weeks; and the remaining 2 after 8 weeks.

Group V.—Coronal partial hypophysectomy was carried out in 12 female rats which were killed after 3 weeks.

Group VI.—Partial hypophysectomy was performed in 15 female rats and mock operations in 14 controls. On the day following the operation, 6 experimental and 7 controls were given 4-methyl-2-thiouracil in drinking water for 14 days, at the end of which time they were killed. The remaining rats were given methyl thiouracil for 14 days, beginning 1 month post-operatively.

RESULTS

In general, apart from cases complicated by sepsis, pneumonia or total hypophysectomy, the body weights after partial hypophysectomy were not significantly lower than those of the mock-operated animals. The experimental animals appeared to eat well, were active and showed no testicular or adrenal atrophy. Mean adrenal weights were 26 mg./100 g. body weight as compared with 25 mg./100 g. body weight in controls.

Normal pituitary/body-weight relationship (Fig. 4)

There was a conspicuous sex difference in the weight of the pituitary gland for rats of equal weight, females showing a greater pituitary body-weight ratio than males. This difference was noted also when absolute pituitary weights were compared for male and female rats of the same age, the figures confirming

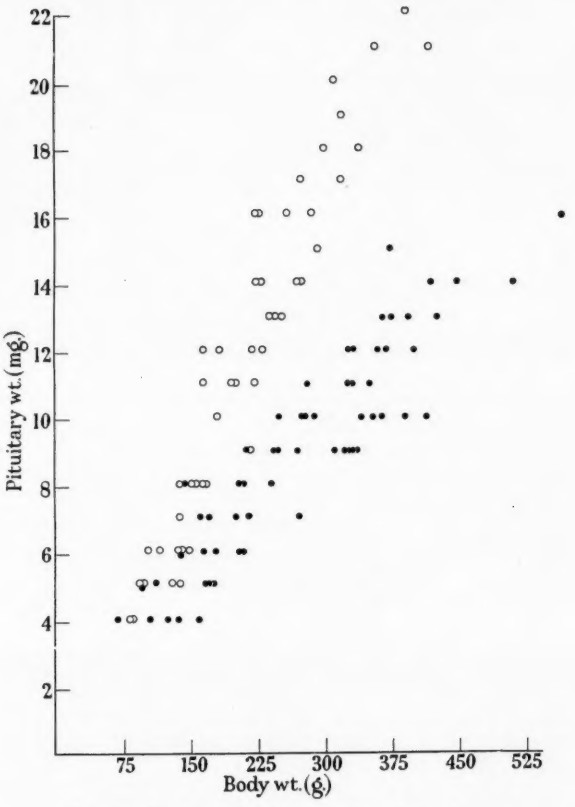


FIG. 4.—Pituitary weights in male and female rats.
○ = females.
● = males.

the findings of Hatai (1913). In the case of the males, the formula for the regression line of Ym (pituitary weight in mg. for males) on Xm (body weight in g. for males) is $Ym = 1{\cdot}727 + 0{\cdot}0264\ Xm$. For females, the formula for the regression line of Yf (pituitary weight in mg. for females) on Xf (body weight in g. for females) is $Yf = 2{\cdot}37 + 0{\cdot}0436\ Xf$.

The amount of anterior pituitary tissue which was resected varied between 1·4 and 2 mg. in the male rats, and was higher (up to 3 mg.) in the larger females.

By subtracting this value from the total, as derived from the regression equations, the weight of remnant could be estimated. In 6 of the rats killed immediately post-operatively, the weight of the remnant estimated in this way corresponded well with its actual weight, when the resected portion was removed in one or two fairly large pieces. In the 5 remaining rats the resected piece fragmented and there was a discrepancy between actual and estimated weights of the

TABLE I.—*Weight of Pituitary Remnant at Varying Times after Sagittal Partial Hypophysectomy*

		Partial hypophysectomy			Controls (sham-operated)		
Post-operative time (weeks)	Sex	Animals (number)	Weight of pituitary mg./100 g. body weight	S.E. (±)	Animals (number)	Weight of pituitary mg./100 g. body weight	S.E. (±)
1	M.	9	2·1	0·06	5	3·6	0·05
3	M.	13	1·8	0·13	7	3·8	0·14
5	M.	5	1·6	0·16	5	3·1	0·22
9	M.	8	1·4	0·02	8	3·3	0·11
2	F.	9	2·6	0·18	9	5·5	0·09
6	F.	10	2·4	0·08	7	5·6	0·09
8	F.	8	2·1	0·20	2	5·2	—

remnant. In such instances, when only minute pieces were recovered, the experiments were excluded from the main data. The estimated mean proportion of pituitary resected was 28 per cent (range 21–42). Since the mean weight of pars nervosa and pars intermedia was equivalent to approximately one-quarter to one-fifth of the total pituitary, the operation represented an average 35 per cent resection of pars anterior. In no case, so far as we could ascertain, was a major proportion resected leaving only a minute fragment of residual tissue.

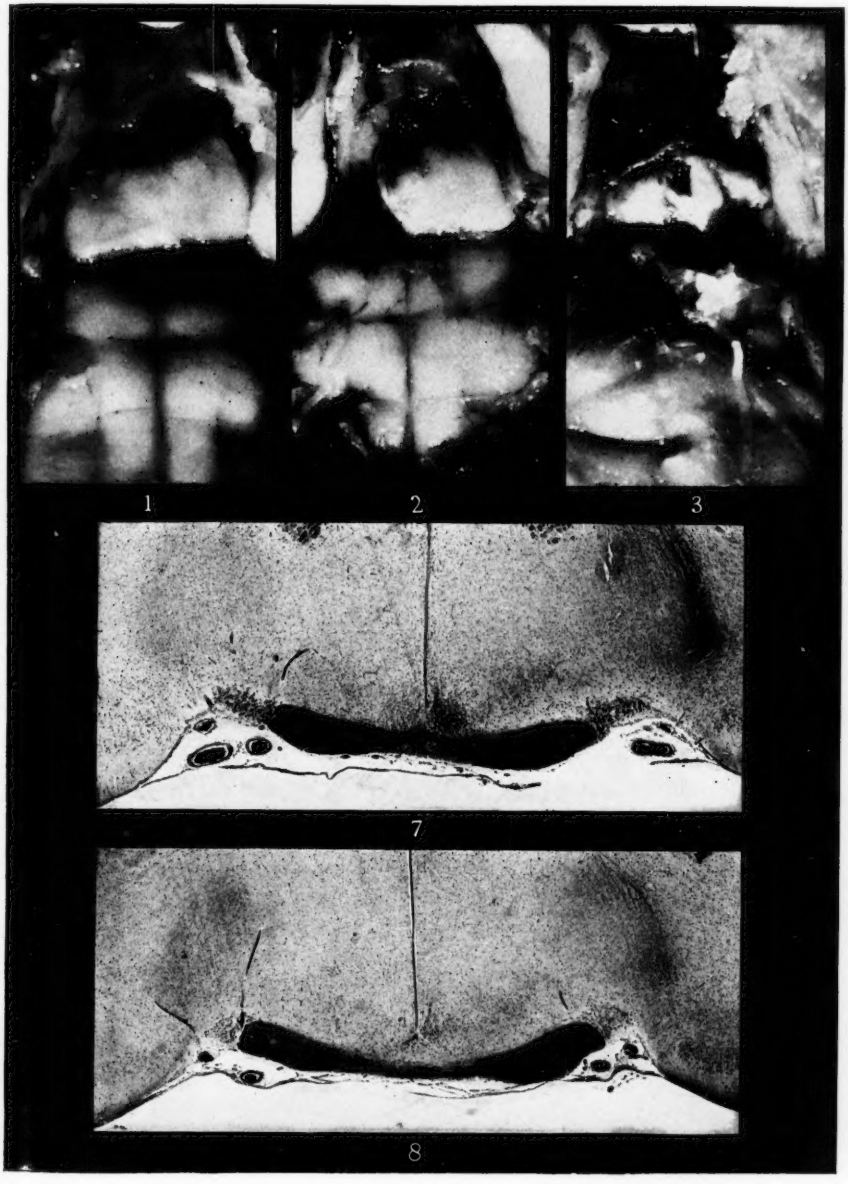
Weight of remnant at intervals after sagittal partial hypophysectomy

The results are summarised in Table I and Fig. 5 and 6.

The weights for the remnants were approximately half those of the controls in both males and females at all the stages examined. Over the longer periods considerable whole body growth occurred and it appears that the pituitary gland or its remnant took part in this somatic growth process at a rate no greater

EXPLANATIONS OF PLATES

FIG. 1.—Ventral view of intact pituitary gland (×8).
FIG. 2.—Similar view after sagittal partial hypophysectomy.
FIG. 3.—Similar view after coronal partial hypophysectomy.
FIG. 7.—Coronal section through hypothalamic region 9 weeks after partial hypophysectomy; H. & E. ×20; shows small area of gliosis in vicinity of tuberal nucleus.
FIG. 8.—Similar lesion in control sham-operated rat.
FIG. 9.—Dorsal view, pituitary 9 weeks after mock operation.
FIG. 10.—Similar view, 9 weeks after sagittal partial hypophysectomy. The pars nervosa can be seen on the right of the photograph.
FIG. 11.—Similar view, 9 weeks after coronal partial hypophysectomy.
FIG. 12.—Section of pituitary remnant in male rat 9 weeks after partial hypophysectomy. PAS Orange G ×830.
FIG. 13.—Section of control gland from sham-operated male rat, same magnification and staining as previous figure; shows similar cytological features.



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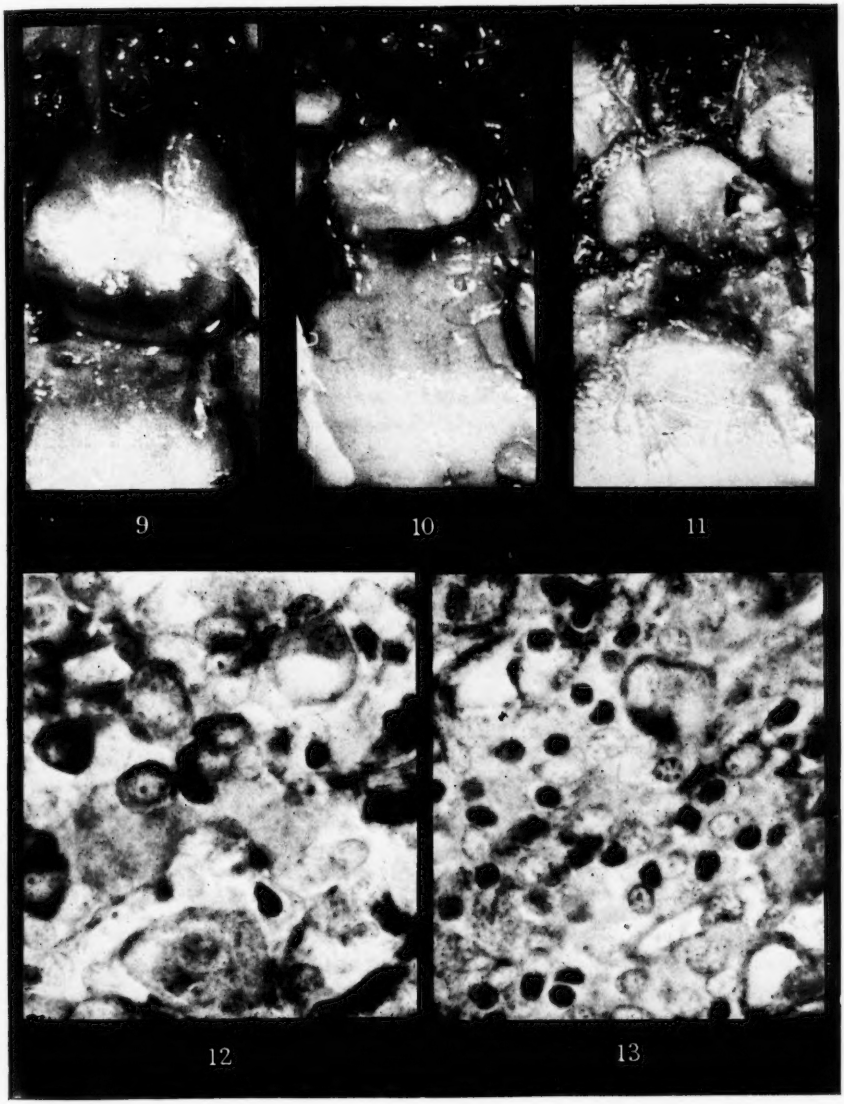

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than that of the whole body. There is no evidence that the remnants increased in mass at a rate greater than that of the intact gland in growing rats.

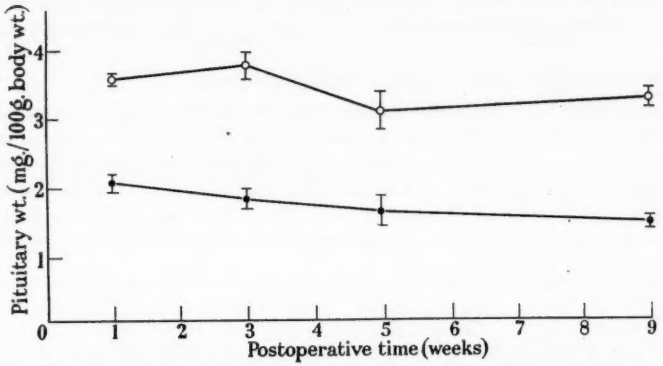

FIG. 5.—Pituitary weights after sagittal partial hypophysectomy in male rats.
● = partial hypophysectomy.
○ = controls.
The vertical lines represent + and − the S.E. of the mean.

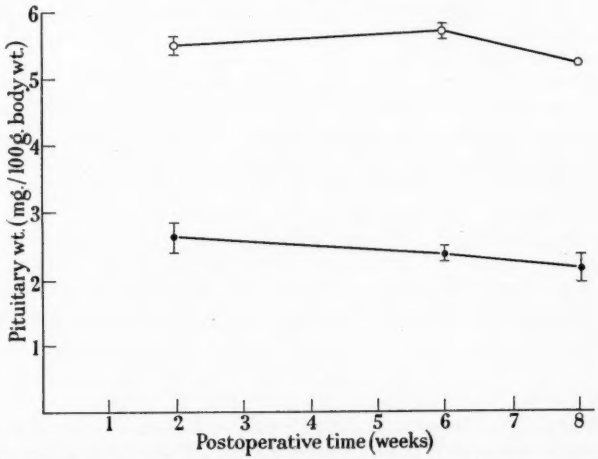

FIG. 6.—Pituitary weights after sagittal partial hypophysectomy in female rats.
● = partial hypophysectomy.
○ = controls.

Examination of hypothalamic nuclei

The inevitable destruction of the hypothalamico-hypophyseal portal vessels on the side of the resection raised the possibility of hypothalamic damage and this was investigated in the serial sections cut coronally through the hypothalamic region. A small lesion consisting of collapse and slight gliosis was seen in a homolateral tuberal nucleus (Fig. 7) in 1 of 5 rats partially hypophysectomized

9 weeks previously, and a similar lesion was found on the contralateral side in another rat. There was a similar lesion in a control sham-operated rat (Fig. 8). It was concluded that there was no significant microscopical abnormality confined to the homolateral side which might have prevented regeneration.

Pituitary weights after coronal partial hypophysectomy

The proportion resected was usually much smaller in this series and varied considerably in weight. While it is therefore not possible to make an assessment of the size of the remnant at the time of operation, the mean-weight after 3 weeks was 3·35 mg./100 g. (± S.E. 0·3) body weight as compared with sham-operated controls (after 2 weeks) of 5·5 mg./100 g. (± S.E. 0·09). These findings show a trend which confirms the results in Series III (Sagittal Partial Hypophysectomy), (Fig. 9–11).

Cell counts

This is an unsatisfactory procedure owing to difficulties in differentiating lightly stained cells. This was partly overcome by examining the PAS Orange G stained sections with phase-contrast microscopy. The counts varied in both remnants and control glands. After 9 weeks, the mean level for α cells was 54·7 per cent (range 45–58); for β cells, 12 per cent (range 10–16); and for chromophobes 34 per cent (range 25–37). Controls measured 56 per cent for α cells (range 52–59); 11 per cent for β cells (range 9·5–12); and 33 per cent for chromophobes (range 29–38).

After a period of 9 weeks, the basophiles were increased in size and often showed the cytoplasmic vacuolation, considered by Reiss *et al.* (1937) to be characteristic of pituitary undergoing regeneration. However, control sham-operated animals of the same age and weight (and also unoperated controls) showed the same cytological variations which should therefore be interpreted as a normal variation with ageing. Acidophiles were not reduced in number as Crooke found in minute fragments left accidentally after attempted hypophysectomy (Fig. 12 and 13). There is a good deal of variation in the cell counts between rats, but no significant difference between the means from partially hypophysectomized and control rats. No increase in mitotic figures was found in the residual tissue.

Goitrogen series

The thyroid weights were not significantly different in the two groups after 14 days of methyl-thiouracil; the mean weight of the thyroid in the partially hypophysectomised rats was 18·6 mg./100 g. body weight and that in the controls 19·5 mg./100 g. body weight. Unstimulated thyroids from unoperated rats of approximately the same weight were 8·8 mg./100 g. Measurements of the cell heights showed a significant variation between experimental and control groups, both immediately after partial hypophysectomy and one month later. In the groups tested immediately post-operatively, the mean thyroid cell height in control sham-operated rats was 16·9 ± S.E. 0·5 μ and that of partially hypophysectomised rats 12·7 ± S.E. 1·1 μ. When the thiouracil was begun 1 month after operation, the controls measured 16·2 ± S.E. 0·4 μ and the experimental rats 13·4 ± S.E. 0·3 μ (Table II).

TABLE II.—*Thyroid Cell Height Measurements after Two Weeks Methyl Thiouracil Administration*

Number of rats	Operation	Post-operative time (weeks)	Mean cell height (μ)	S.E.
6	Partial hypophysectomy	0–2*	12·7	±1·1
7	Sham operation (control)	0–2*	16·9	±0·5
9	Partial hypophysectomy	4.6†	13·4	±0·3
7	Sham operation (control)	4–6†	16·2	±0·4

* Methyl-thiouracil started immediately after operation.
† Methyl-thiouracil started 4 weeks after operation.

DISCUSSION

The results in this series of experiments provide no evidence of regeneration in the pituitary remnants up to 9 weeks after partial resection. This confirms the opinion of Smith (1930) who did not compare resected and residual weights, but was able to determine the proportion of surviving tissue which was compatible with certain functions. The view advanced by Cameron (1952) that powers of regeneration are present to some degree, and Reiss *et al.* (1937) that regeneration is remarkably rapid and complete, cannot be confirmed. Cameron cites as evidence the incidence of mitosis in the gland of small animals (Hunt, 1942) and after endocrine manipulations, such as administration of oestrogens (Wolfe, 1935); this process may not be controlled by the same factors as regeneration. The incidence of mitosis, in fact, may not be a reflection of the ability of the gland to regenerate, but may represent an increased turnover of cells consequent upon the physiological stimuli.

The present findings show a major disagreement with the report of Reiss *et al.*, who claimed that 1 month after partial resection, the pituitary remnant was indistinguishable from a normal control pituitary. These observations were made without any measurements or weights and the visual impression of the size of this small soft gland may be misleading. In the work of Reiss *et al.*, microscopic verification does not appear to have been done in every case, and fibrin entwined with the remnant or leucocytic infiltration following infection may be further complicating factors tending to increase the mass of the pituitary. Further, from the description of their procedure, these authors probably removed a sagittal half of the anterior pituitary when they removed a substantial portion, and it is very difficult to picture the hypertrophied half of a gland appearing indistinguishable from normal in these circumstances. For this to be the case, regeneration would have had to take place from the cut surface, a most unlikely event in a mammal whereas, in the event of a possible hypertrophy involving the whole remnant, a mis-shapen asymmetrical gland should be anticipated. In their Table I, it is evident that they did not distinguish between male and female rats; the great sex variation in hypophysis weight could probably account for some difference.

Certain cytological changes have been described in the remnant after partial hypophysectomy. Reiss *et al.* (1937) describe enlargement and vacuolation of the β cells, changes which have been found in the present series of experiments in both partially hypophysectomised and control sham-operated rats and in unoperated rats of the same age. Similar changes are described in normal old

rats (Severinghaus, 1938) and it seems likely that these changes are within normal variations. The cytological alterations described by Crooke (1938) which include diminution in number or complete absence of acidophiles have not been found in remnants of the present series. There are major differences between the type of remnant in Crooke's animals and the present series. First, the remnants in Crooke's cases were minute, all but one of the fragments measuring less than 1 mm. in all directions. In 2 rats, most of tissue examined by Crooke was described as forming a crescent on the infundibulum and may have represented mainly pars tuberalis. In the other cases the positions of the fragments were not described, and in these the vascular connections with the hypothalamus may possibly have been disrupted. Acidophiles are reported to be absent from the anterior lobe of the pituitary of rats when the gland has been transplanted to the anterior chamber of the eye (Westman and Jacobsohn, 1940) and a reduction of acidophiles follow thyroidectomy (Cameron). The absence or reduction of acidophiles may possibly therefore be related to altered hormonal relationships, which may account for the difference in observations.

It is difficult to reconcile the present findings with the views of Harris (1955) in studies on the anterior pituitary after stalk section. Necrosis of the anterior lobe follows section of the stalk in rats (Barrnett and Greep), sheep (Daniel and Prichard) and man (Russell, 1956). Whether this occurs in the rabbit is uncertain, and Harris has worked mostly on rabbits. After stalk section in this species, good function is re-established provided there is no obstruction to the proliferation of portal blood vessels (Harris, 1955). It is known that there is an independent arterial supply to the anterior pituitary of the rabbit and it is therefore unlikely that extensive necrosis follows stalk section. In the rat, however, Harris (1950) reported a similar recovery of function after stalk section and only one inconstant artery has been described in this species (Landsmeer, 1951). Barrnett and Greep (1951) described only a peripheral rim of surviving cells after stalk-section in rats and Daniel and Prichard confirmed the presence of necrosis with a slightly greater proportion of surviving tissue. Cheng, Sayers, Goodman and Swinyard (1949) reported resumption of ACTH discharge after stalk section, but there are no microscopical preparations to confirm the presence of necrosis. Daniel and Prichard (1957) raised the possibility of regeneration of the anterior pituitary to account for these instances of functional recovery, but no evidence could be found to support this hypothesis. What appears to be most likely is that the proportion of remaining pituitary tissue is probably sufficient for resumption of oestrus cycles and ACTH discharge in the rat, Smith (1930) having shown that anterior lobe function can be detected with as little as 10 per cent of anterior pituitary present. In any case, interruption of the stalk may not provide the same conditions that may follow partial hypophysectomy with an intact hypothalamic-hypophyseal portal system. In the former instance most hormones will pass to the anterior pituitary directly, whereas in the latter condition, most of the hormones reaching the anterior lobe will first have passed through the hypothalamic nuclei where possible metabolic alteration may take place. It may therefore not be justifiable, on these hypothetical grounds, to compare partial hypophysectomy and necrosis induced by stalk-section as precursors of regeneration.

The fact that the remnant was able to take part in the normal growth process, that is it increased in mass at the same rate as the normal gland, suggests that

the process of regeneration may be different from mechanisms involved in normal somatic growth.

We noted that, in general, partially hypophysectomized rats were not distinguishable from controls on clinical grounds nor on the basis of body or adrenal weight changes. The induction of goitres by 4-methyl-2-thiouracil provided a means for differentiating between partially hypophysectomized and control rats and we found that this difference persisted for at least one month after operation. Thyroid follicular cell height has been shown to increase with increasing amounts of circulating thyrotrophic hormone (Rawson and Starr, 1938; Astwood *et al.*, 1943). The thyrotrophic hormone secretion evoked by the dosages used in this experiment was reflected in a well-marked increase both in thyroid weight and in thyroid follicular cell height.

In view of the differences noted in the thyroids from rats with intact pituitary glands and those with pituitary remnants, it seems likely that the level of TSH may represent what is close to the maximal secretion of the particular gland stimulated.

Greer (1952) has suggested that thiouracil induced goitres may be partly prevented by hypothalamic lesions, but this is not considered to be a factor in this series in view of the insignificant hypothalamic alterations found on examination of serial microscopic sections.

SUMMARY

After partial resection of the anterior lobe of the pituitary gland in rats, no evidence of regeneration was found in the remnant.

After 14 days on 4-methyl-2-thiouracil, which was used to stimulate thyrotrophin secretion, the thyroid follicular cell heights were significantly different in rats with intact and half pituitaries. After one month, this difference was still present. There is therefore no evidence of functional adaptation on the part of the pituitary remnant, when tested in this way.

We wish to thank Professor D. S. Russell for providing facilities and offering helpful criticism during the course of this work. We are grateful also to Mr. K. V. Swettenham for microscopical preparations, Mr. A. L. Gallup for the photographs, Mrs. M. V. Whittle for technical help, and to Dr. S. L. Morrison for help with the statistics.

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THE SIGNIFICANCE OF RENAL GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN EXPERIMENTAL HYPERTENSION IN THE RAT (A HISTOCHEMICAL STUDY)

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ALTHOUGH there is general unanimity concerning the importance of renin, the humoral pressor principle released by the ischaemic kidney to induce hypertension, its site of formation remains the object of much speculation. Since Goormaghtigh (1937) postulated an activity of the granulated cells of the preglomerular arteriole, first described by Ruyter (1925), changes in these juxtaglomerular cells have been implicated in the aetiology of various forms of experimental hypertension. Recent studies by Hartroft and Hartroft (1953), and by Tobian, Thompson, Twedt and Janacek (1958), have firmly established a relationship of the stainable juxtaglomerular granulations to hypertension. No direct connection of the juxtaglomerular cells to a known humoral factor, however, could be demonstrated.

Another structure, the macula densa of Zimmermann (1933), which is intimately associated with the vascular pole of the glomerulus, escaped notice in studies of experimental hypertension because only severe degenerative alterations could be seen by ordinary histological methods.

A special functional significance of this segment of the distal tubule was inferred by McManus (1944) because of the basal position of the Golgi element in the cells composing it. This arrangement is the reverse of that found in the other cells of the distal convolution. Electron microscopic studies by Rhodin (1958), however, failed to demonstrate any special features in the ultrastructure of the macula densa, which could distinguish this segment from adjacent distal tubule cells.

Using a sensitive histochemical technique for the demonstration of coenzyme-linked dehydrogenase activities (Hess, Scarpelli and Pearse, 1958*a*, *b*) a strong reaction for the triphosphopyridine nucleotide-dependent glucose-6-phosphate dehydrogenase can be demonstrated selectively in the macula densa of rat kidney. A high activity of 6-phosphogluconate dehydrogenase, which is also localized selectively in the macula densa, had been noted independently by Nachlas, Walker and Seligman (1958) using a similar histochemical method.

The present report concerns the finding of a direct relationship between the enzymic activity of the macula densa and renal ischaemia resulting in hypertension. This process is independent of the preservation of the excretory tubular apparatus.

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MATERIALS AND METHODS

Twenty-three female rats (hooded strain, 150–230 g.) were operated on according to the method of Wilson and Byrom (1941).

Hypertension was produced by constricting the right renal artery by a silver ribbon clip, the left kidney remaining untouched. The animals were fed on a diet containing one per cent NaCl and had free access to tap water. Systolic blood pressure was measured in the lightly anaesthetized animals by Floyer's (1951) modification of the method described by Byrom and Wilson (1938) using a tail plethysmograph. The pre-operative blood pressure ranged from 95–125 mm. Hg (mean 114 mm. Hg).

In three rats of this series, the right kidney was excised 26, 51 and 52 days after the clip had been applied to the renal artery. These animals were sacrificed 31, 13 and 18 days after unilateral nephrectomy.

Controls included untreated stock rats of the same weight and sex as well as two " sham-operated " animals. The hyperplastic kidneys of 3 unilaterally nephrectomized animals were also examined.

The rats were killed by coal-gas 10–110 days after unilateral clamping of the renal artery. Both kidneys were quickly removed and cut into 3 parts. The tissue block out of the middle third of the organ was quenched on a metal chuck by immersion of the chuck only in a mixture of acetone and dry ice. Fresh frozen sections were cut at 8 μ in a remotely controlled cold microtome (cryostat), mounted on clean coverslips and covered with the incubating medium. Glucose-6-phosphate dehydrogenase, and diphosphopyridine and triphosphopyridine nucleotide diaphorase activities, were demonstrated by the methods of Hess, Scarpelli and Pearse (1958*b*) and Scarpelli, Hess and Pearse (1958) using an *N*-thiazol-2-yl-substituted tetrazolium salt as electron acceptor in the presence of cobaltous ions. For the demonstration of 6-phosphogluconate dehydrogenase activity, glucose-6-phosphate disodium salt was replaced by the barium salt of 6-phosphogluconic acid. This reaction required 75 min. incubation time because of the low solubility of the substrate, whereas in the case of glucose-6-phosphate dehydrogenase the incubation time was 30 min. at 37°. Non-specific esterase activity was shown in cryostat sections, fixed in formol-calcium for 1 min. at 20°, by the method of Holt and Withers (1958), using *o*-acetyl-4-chloro-5-bromoindoxyl as a substrate. For tissue morphology acetic-ethanol fixed cold microtome (cryostat) sections were stained by the acid Solochrome cyanine method (Pearse-1957) and also by the methyl green-pyronin method (Kurnick, 1955). Sections from paraffin-embedded tissue were stained with haemalum and eosin.

RESULTS

Unilateral constriction of renal artery

Hypertension developed in all the experimental animals. The blood pressure readings before the animals were sacrificed and the relative activity of glucose-6-

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EXPLANATION OF PLATE

FIG. 1.—Macula densa of distal tubule from normal control rat. Glucose-6-phosphate dehydrogenase in mitochondria mainly in basal portions of the cells, facing the glomerulus. ×1170.

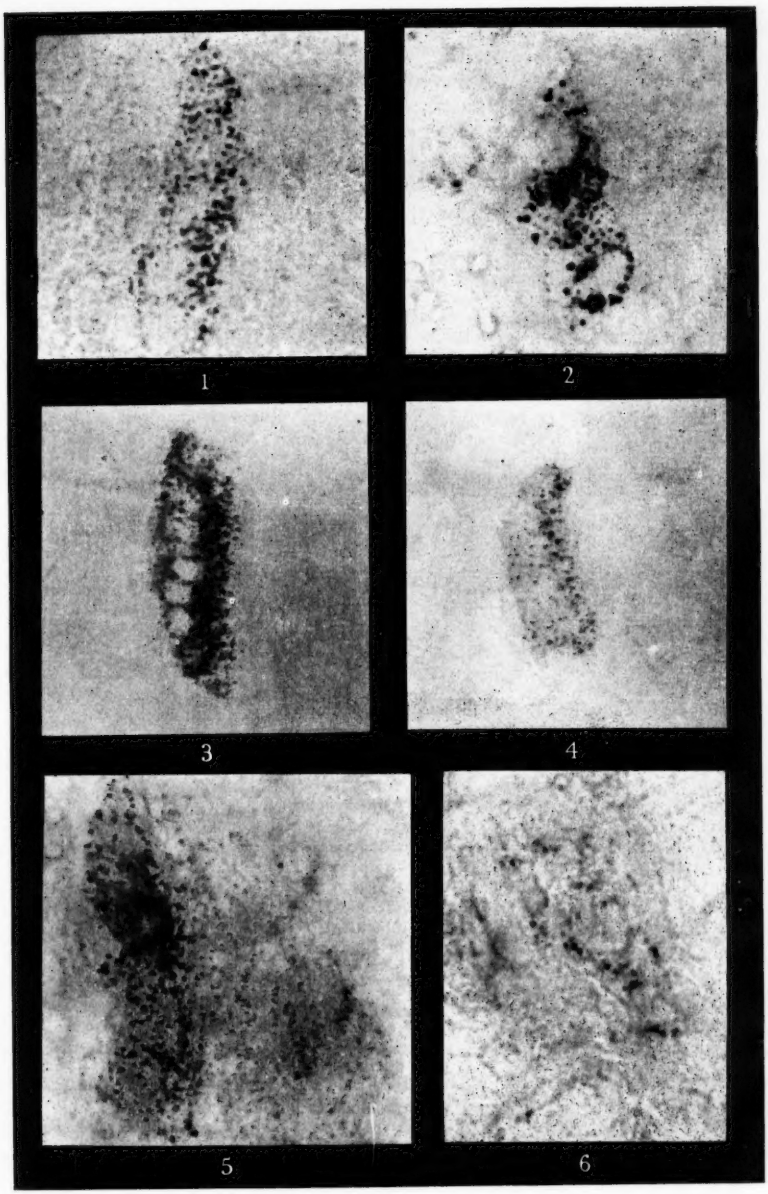
FIG. 2.—Rat No. 1. Macula densa shows patchy mitochondrial swelling and enlarged cells in the clamped kidney, 10 days after operation. Gross fatty change in neighbouring tubules. Glucose-6-phosphate dehydrogenase. ×1170.

FIG. 3.—Rat No. 3. Moderate increase of glucose-6-phosphate dehydrogenase activity in macula densa from the clamped kidney, 18 days after operation. ×1400.

FIG. 4.—Same animal as Fig. 3. Decrease of enzymatic activity in macula densa of the untouched kidney. Glucose-6-phosphate dehydrogenase. ×1170.

FIG. 5.—Strong activity of glucose-6-phosphate dehydrogenase in macula densa from clamped kidney 30 days after operation (animal No. 7). Moderate activity in juxtaglomerular cells situated in the angle between the macular segment and the glomerular capsule. ×1170.

FIG. 6.—Same section as Fig. 5. Activity in juxtaglomerular cells which are excentrically localized within the wall of the afferent arteriole at the glomerular root. ×1170.



Hess and Pearse.

phosphate dehydrogenase in maculae densae of both the clamped and the contralateral, untouched, kidneys are listed in the Table. In a normal rat, this enzymic activity of the macular segment has an intramitochondrial localization and is concentrated mainly in the basal portion of the cytoplasm facing the glomerular root (Fig. 1). Up to 10 of these active columnar cells may be present in the plane of the tissue section, representing the entire width of the disciform macula. The activity of 6-phosphogluconate dehydrogenase was found to run parallel to that of glucose-6-phosphate dehydrogenase and is therefore not especially mentioned hereafter.

TABLE

Animal No.	Days after clamping	Blood pressure (mm. Hg)	Glucose-6-phosphate dehydrogenase activity of macula densa†	
			Clamped kidney	Untouched kidney
1	10	135	++	++
2	10	140	+++	++
3	18	160	+++*	+
4	23	155	+++*	+
5	23	178	+++*	+
7	30	194	+++*	+
8	30	175	+++	++
9	30	205	+++	++*
10	33	168	+++*	++
11	35	162	+++	+
12	36	160	+++	++
13	36	158	+++	++
14	40	170	+++	++
15	49	175	+++*	+
16	49	190	+++*	++*
19	67	165	+++	+
20	97	182	+++*	++*
21	100	176	+++	+
22	110	172	+++*	+
23	110	180	+++*	+
25 "sham"-operated	35	117	++	++
26 "sham"-operated	35	115	++	++

* Reaction in juxtaglomerular cells.

† Estimated as the mean activity of reactive cells in macular segments throughout the cortical zone; +++ = strong activity, ++ = moderate activity, + = weak activity.

Ten days after clamping of the renal artery, changes in the enzymic activity of the maculae in the ischaemic kidney had already become apparent. The columnar cells were slightly larger and they contained some swollen mitochondria, seen in sections as an increase in the size of the sites of enzymic activity (Fig. 2). By contrast, gross mitochondrial swelling and an accumulation of fine fat droplets was present throughout the tubules of the cortex. These ischaemic alterations were shown clearly by the diaphorase reactions, in confirmation of the findings of Mustakallio, Saikkonen and Mustakallio (1956), obtained by a less sensitive method. The untouched kidney was not visibly altered.

From 18 days after operation onwards, highly active macular segments were seen in the clamped kidney without marked mitochondrial swelling (Fig. 3). In the contralateral kidney, the macular cells showed a decrease in mitochondrial activity of glucose-6-phosphate dehydrogenase (Fig. 4).

In the later phases of hypertension, this activity of the maculae densae in the ischaemic kidney was even more pronounced. Figure 5 shows the macula of a clamped kidney 30 days after operation in an animal with a blood pressure of 194 mm. Hg. The cells are clearly enlarged and stuffed with small mitochondria. In the contralateral kidney, however, the corresponding cells were almost non-reactive. In animals with hypertension sustained for a longer period, activity of glucose-6-phosphate dehydrogenase in the untouched kidney increased once more and reached almost normal intensity. This rise in activity, however, was never uniform and could be related to patchy ischaemic areas in the cortical zone due to hypertrophic changes of the arteries and arterioles. Maculae situated in well vascularized areas still showed decreased activity.

The preservation and increase of glucose-6-phosphate dehydrogenase activity in the maculae of the clamped kidney was regularly observed up to 110 days after operation. It was independent of the level of blood pressure and was not related to the degree of tubular atrophy. The fine mitochondrial pattern was well preserved even in a few instances of almost complete destruction of the excretory tubules. The survival of the macula in these cases points strongly to an autonomous function of this tubular segment and suggests that it may be vascularized independently of the result of the distal convolution. Alternatively, the fact that its main respiratory effort is apparently made *via* the hexose monophosphate shunt may mean that it is able to function relatively independently of the local oxygen tension. In confirmation of the earlier studies by Wilson and Byrom (1941) we found the degree of atrophy of the clamped kidney to be subject to variation and not related to the degree of hypertension obtained.

In ten of the hypertensive animals glucose-6-phosphate dehydrogenase activity was observed in the juxtaglomerular cells of the clamped kidney (Figs. 5 and 6). This activity was comparatively weak and related directly to an increase of enzymic activity in the smooth muscle cells and endothelium of hypertrophic interlobular arteries and glomerular arterioles. This activity was also observed in the endothelial cells of the tufts and capsules of the glomeruli undergoing fibrosis. In 3 cases, the same activity was seen in the untouched kidneys, again related to hypertrophic vascular changes and, in one instance, it was accompanied by fibrinoid necrosis of arteries. Increase of glucose-6-phosphate dehydrogenase activity in the juxtaglomerular cells was accompanied by an increase of diphosphopyridine nucleotide diaphorase activity. This was not noted in the case of the macula densa.

Although no change in distribution of the two diaphorases was observed in either kidney in the hypertensive animals, glucose-6-phosphate dehydrogenase activity was occasionally increased in the tubular zone of the inner cortex and also in the proximal convolutions. These changes are similar to those seen by us in the kidney of potassium deficient rats and they are probably due to electrolyte disturbances. No change could be observed in the normally weak esterase activity, or in the pyroninophilia of the macular segment.

Excision of the clamped kidney

Removal of the clamped kidney in three animals with hypertension of 26–52 days duration resulted in maintenance of elevated blood pressure (165–176 mm. Hg). The untouched hyperplastic kidney, examined after the preoperative

blood pressure was re-established, showed a high glucose-6-phosphate dehydrogenase activity of the maculae, together with gross hyperplastic alterations in the arteries and arterioles. The enzymic activity of the macular segment approached the high activity observed in the excised clamped kidney.

Controls

Two " sham-operated " animals remained normotensive and developed a moderate unilateral fibrous perinephritis. Glucose-6-phosphate dehydrogenase activity of maculae densae of both kidneys corresponded to that of untreated animals. Furthermore, no deviation from the normal activity of the macula was observed in the hyperplastic kidneys after unilateral nephrectomy in animals exhibiting a blood pressure of 135–140 mm. Hg.

DISCUSSION

The present experiments demonstrate that, in the rat, renal ischaemia resulting in hypertension is accompanied by a high activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in the maculae densae of the ischaemic kidney. This enzymic activity is independent of degenerative changes in the excretory tubules and is consistent with an almost autonomous structural preservation of the macular segment. These findings suggest a secretory activity of the macula densa and we believe that they may be related to the formation of a pressor principle.

The relatively strong enzymic activity of the macula in the normal kidney is compatible with the fact that renin (or its precursor) is a normal constituent of the renal cortex. Recent studies by Peart, Gordon, Cook and Pickering (1956) indicate that, in rabbit kidney tissue, renin is closely associated with cortical areas containing glomeruli but is apparently not linked to the glomerular structure itself.

Following restriction of blood flow through the kidney, renin is released into the renal vein (Kohlstaedt and Page, 1940 ; Huidobro and Braun-Menendez, 1942) and it is required in amounts equal to three times the normal kidney content per day to maintain an elevated systemic blood pressure, as shown by Blacket, Depoorter, Pickering, Sellers and Wilson (1950) in perfusion experiments. Although renin has been successfully demonstrated in the blood of dogs only in the first phase of renal hypertension (Haynes and Dexter, 1947), its presence in the later stage can be concluded from a lowering of blood pressure observed after administration to dogs of antibodies against renin (Wakerlin, Bird, Brennan, Frank, Kremen, Kuperman and Skom, 1953).

The physiological role of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in a possible formation of the enzyme renin must now be considered. The significance of the hexose-monophosphate pathway, to which these dehydrogenases belong, is complex since the intermediates can follow very different metabolic routes (Racker, 1954 ; Horecker and Mehler, 1955). The direct oxidative utilization of glucose results in the intermediate formation of pentoses, which can be used for the synthesis of nucleic acids. A close relationship between ribonucleic acid content and hexose-monophosphate levels in tissues has been demonstrated by Glock and McLean (1955). A comparatively high activity of

glucose-6-phosphate dehydrogenase has been observed in proliferating and malignant tissues by Dickens (1955). Histochemically, this finding was confirmed by the demonstration of a high glucose-6-phosphate dehydrogenase activity in the growing edge of experimental malignant tumours (Pearse, 1958). Ribonucleic acid, formed from pentoses produced *via* the hexose-monophosphate shunt, may serve in growing tissues as well as in the macula densa as templates for the synthesis of enzymes (Dixon and Webb, 1958) or other proteins. The decrease of glucose-6-phosphate dehydrogenase activity in the macular segments of the untouched kidneys before the onset of vascular alterations may be due to competitive inhibition brought about by the strong activity in the clamped kidney or to the mechanical effects of increased arteriolar pressure.

Although the macula densa has a high activity of glucose-6-phosphate dehydrogenase, in common with elements of the autonomous nervous system (Hess, Scarpelli and Pearse, 1958*a* and *b*) it lacks the high cholinesterase activity of the latter and, therefore, cannot be related to a cholinergic system. On the other hand, it is unlikely that pressor amines are formed in the ischaemic kidney because according to Blacket, Pickering and Wilson (1950), infusions of adrenaline and noradrenaline fail to produce sustained hypertension.

High enzymic activity in a specialized part of the clamped kidney cannot be explained by the theory of Grollman (1954) that renal ischaemia causes deficiency of a renal depressor mechanism released by the normal kidney. Our findings show that even in later phases of renal hypertension a high enzymic activity, confined to the macula densa, is maintained in the ischaemic kidney. The action of an extra-renal pressor mechanism of the type described by Floyer (1955) is not, however, excluded at this stage.

Enzymic activity demonstrated in the juxtaglomerular cells was closely related to the activity in vascular smooth muscle and endothelium. This strongly suggests that the juxtaglomerular cells are vascular in origin. No attempt was made to correlate enzymic activity with juxtaglomerular granulations. In general our findings support the possibility that the vascular tree may be directly implicated in the development of hypertension and that it may even, as suggested by Pasqualino and Bourne (1958) be capable of secreting a substance which affects the clamped kidney.

SUMMARY

In rats rendered hypertensive by unilateral clamping of the renal artery, histochemical methods show a high activity of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in the macula densa of the clamped kidney. This activity, which is moderately strong in the kidney of untreated animals, was markedly decreased in the macular segments of the contralateral, untouched kidneys from hypertensive rats, provided that no obliterating vascular changes were present. Excision of the clamped kidney in chronically hypertensive rats, resulting in the maintenance of elevated blood pressure, was followed by an increase of activity in the macula densa of the remaining kidney and also by marked ischaemic alterations.

The results reported indicate that enzymes of the hexose-monophosphate pathway, present in the macular segment of the distal tubule, are increased following renal ischaemia. It is suggested that this enzyme system is implicated in the formation of a renal pressor principle.

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BIOCHEMICAL STUDIES ON EXPERIMENTAL CARDIOPATHY: ELECTROLYTES IN RAT TISSUES*

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EARLIER publications from this Institute (Selye and Mishra, 1958; Selye and Renaud, 1958) have been concerned with the description of an experimental cardiac lesion produced by exposure of the rat to various steroids and electrolytes (Electrolyte-Steroid Cardiopathy). These lesions consisted of massive, necrotic patches. They differed from the usual spontaneous infarcts, inasmuch as they were not due to histologically detectable vascular occlusions (Selye and Mishra, 1958). Recently reviews have been made of the function and participation of electrolytes in cardiac disease (Overmann, 1951; Manery, 1954). In an attempt to elucidate the biochemical mechanisms responsible for this cardiopathy, we have studied the distribution of the most important body electrolytes in 6 tissues of the rat, and the present work describes these findings.

MATERIALS AND METHODS

Animals.—The animals used were female Sprague-Dawley rats, with an initial weight of 90–105 g. They were fed Purina Fox Chow and water *ad libitum*.

Preparation of samples.—The animals were killed by ex-sanguination performed by cutting the jugular vein. The blood was received directly into siliconed conical tubes and centrifuged for 15 min.; the subsequent analyses were carried out on serum.

The abdominal muscle, heart, kidney, liver and brain were excised and weighed in a preweighed glass-stoppered vessel containing 5 ml. of 10 per cent trichloroacetic acid (TCA). These tissues were then cut into small pieces and the resulting suspension was homogenised in a 50 ml. stainless steel Waring blender with an additional 15 ml. of TCA for 4–6 min. The homogenates were centrifuged for 15 min. in polyethylene tubes and supernatants were analysed for Na, K, Mg, Ca and inorganic phosphate.

Analytical methods.—Na and K were determined directly in suitable dilutions of serum or tissue extracts by the usual method of flame spectrophotometry (using a Beckman Model DU).

Inorganic phosphate was determined by the method of Fiske and Subbarow (1925).

Chloride determination was carried out on the serum, using the method of Schales and Schales (1941). Tissue extracts were prepared for Cl determination as described, except that they were deproteinised with 20 ml. of 80 per cent ethanol instead of TCA. They were then assayed for Cl by the Volhard-Arnolds' method.

Magnesium was determined by complexometry using Eriochrome Black T as the indicator (Schwarzenbach and Gysling, 1949).

Calcium was analysed by a complexometric method described for non-biological specimens by Diehl and Ellingboe (1956). It had to be adapted for Ca determination in biological materials as follows: *Serum:* A volume of 0·1 ml. of serum was pipetted into a 50 ml. beaker containing 5 ml. of 3N KOH and a few drops of Calcein indicator (4 mg. per cent in

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0·25N KOH). The titration was carried out with Complexone IV until a pink colour was obtained. *Tissues :* The technique was identical with that used for the serum, except that, here, 1 ml. of TCA supernatant was used, instead of 0·1 ml. of serum.

EXPERIMENTAL

Four groups of 20 rats were used. The first group acted as controls. All the animals in the second group received 2 mM of NaH_2PO_4 in 4 ml. of water twice daily by stomach tube. The rats of the third group were injected subcutaneously once daily with 100 μg. of 2α-methyl-9α-chlorocortisol acetate (Me–Cl–Col–Ac), suspended in 0·2 ml. of water. The rats in the fourth group received both NaH_2PO_4 and Me–Cl–Col–Ac as above. All treatments were continued for 9 days. On the 9th day all the animals were starved, but had free access to water. After 24 hr. they were killed and processed as previously described. The hearts of the rats in groups I, II and III were normal, whereas most of those in group IV showed various degrees of necrosis.

RESULTS AND DISCUSSION

It is well known that mineralocorticoids such as deoxycorticosterone have a profound Na and Cl retaining- and K excreting-action in experimental animals (Selye and Dosne, 1941 ; Miller and Darrow, 1941) and in humans (Fourman, Kapler, Reifenstein and Dempsey, 1949). Therefore, it is easy to understand that in our experimental conditions the corticosteroid-treated groups (groups III and IV) showed a depletion of K and an increase of Na in the muscle (Table I), since Me–Cl–Col–Ac, used in the experiments, is known to possess both mineralo- and gluco-corticoid activity (Selye, 1955–56). It is not known whether these electrolyte changes have some bearing on the production of cardiac necroses, although moderately low K and high Na have been observed in the muscle in the case of advanced heart failure (Stein, Schwartz and Mersky, 1954).

However, it is established that the feeding of K- or Mg-deficient diets results in cardiac necroses (Selye and Bajusz, 1958 ; Follis, 1942 ; Thomas, Mylon and Winternitz, 1940). Furthermore, if either $MgCl_2$ or KCl is administered simultaneously with phosphate-steroid treatment, no cardiac necrosis can be detected (Selye and Mishra, 1958 ; Selye and Renaud, 1958). These findings suggested that our infarctoid lesion was possibly associated with lowered cardiac values for either K or Mg. In fact, under our experimental conditions, in group IV, the K-content generally did not decrease in the heart (Table I), but the level of Mg dropped considerably in that organ (Table II). It is of interest to contrast the unchanged K levels in the cardiac lesion observed in this experiment with the decrease of K in damaged myocardium reported by Buff (1949). Similar low concentrations of K in the heart have also been described in cardiac diseases (Harrison, Pilcher and Ewing, 1930 ; Wilkins and Cullen, 1933). Whether or not the Mg decrease in the heart is specific of necrosis will now be examined.

Although both phosphate and Na were administered (as NaH_2PO_4) only the Na level increased in the muscle and heart of the electrolyte-steroid-treated group (Table I), no such increase was observed in the case of inorganic phosphate, in fact, there was a sizable decrease of this anion in the muscle of group IV (Table III). Metabolic experiments are being carried out in an attempt to explain the fate of this phosphate ; the results of which will be published later. Although

TABLE I.—*Distribution of Na and K in Various Rat Tissues*

Group	Treatment		Sodium (mEq./kg.)						Potassium (mEq./kg.)					
			Serum	Muscle	Heart	Liver	Kidney	Brain	Serum	Muscle	Heart	Liver	Kidney	Brain
I	None	Av.	158	40·7	55·1	47·0	70·1	52·3	5·72	118·4	78·[illegible]	111·8	77·2	107·7
		S.E.	±1·3	±1·2	±1·2	±2·8	±3·9	±0·9	±0·2	±2·6	±2·6	±4·1	±2·5	±2·8
II	NaH_2PO_4	Av.	153·6	37·0	59·5	47·9	72·0	52·4	5·58	114·9	83·5	113·9	79·1	107·6
		S.E.	±1·9	±1·4	±1·8	±4·6	±3·8	±1·2	±0·2	±2·4	±3·7	±5·5	±3·8	±2·6
III	Me–Cl–Col–Ac	Av.	157·8	*48·8*	58·9	46·5	72·1	51·3	*4·31*	*101·8*	80·5	120·8	76·9	112·1
		S.E.	±1·7	±1·3	±3·0	±3·1	±4·0	±2·1	±0·12	±4·0	±3·6	±9·7	±3·2	±2·7
IV	NaH_2PO_4 +Me–Cl–Col–Ac	Av.	162·5	*57·4*	*69·0*	56·6	74·2	53·0	*3·88*	*100·9*	81·1	131·3	74·7	102·6
		S.E.	±1·9	±2·1	±2·4	±3·7	±4·1	±1·4	±0·10	±3·0	±2·0	±7·5	±3·9	±2·5

Values in italics proved to be statistically significant ($P < 0·05$) as compared with the corresponding normal values (Group I).

TABLE II.—*Distribution of Ca and Mg in Various Rat Tissues*

Group	Treatment		Calcium (mEq./kg.)						Magnesium (mEq./kg.)					
			Serum	Muscle	Heart	Liver	Kidney	Brain	Serum	Muscle	Heart	Liver	Kidney	Brain
I	None	Av.	5·03	3·79	22·5	11·9	13·0	6·2	2·45	22·1	30·4	34·5	15·9	4·6
		S.E.	±0·07	±0·26	±2·2	±1·0	±2·2	±0·5	±0·13	±1·0	±1·4	±1·3	±1·6	±0·26
II	NaH_2PO_4	Av.	*4·31*	4·61	31·2	13·7	16·6	6·3	*3·71*	22·8	32·5	38·5	24·8	4·6
		S.E.	±0·06	±0·27	±4·2	±2·0	±2·8	±0·7	±0·36	±1·0	±2·1	±1·3	±3·2	±0·39
III	Me–Cl–Col–Ac	Av.	*2·98*	4·43	*33·2*	18·2	17·7	5·7	2·63	23·0	*23·2*	36·5	14·9	3·9
		S.E.	±0·14	±0·37	±2·6	±2·2	±3·7	±0·6	±0·21	±1·6	±1·8	±1·0	±3·4	±0·21
IV	NaH_2PO_4 +Me–Cl–Col–Ac	Av.	*2·20*	*5·93*	*35·1*	24·9	*32·9*	6·1	*1·33*	19·5	*15·0*	37·8	16·0	4·4
		S.E.	±0·12	±0·51	±4·0	±2·8	±3·0	±0·7	±0·12	±1·3	±1·1	±2·2	±3·8	±0·08

Values in italics proved to be statistically significant ($P < 0·05$) as compared with the corresponding normal values (Group I).

TABLE III.—*Distribution of Cl and PO_4 in Various Rat Tissues*

Group	Treatment		Chloride (mEq./kg.)			Phosphate (mEq./kg.)					
			Serum	Muscle	Heart	Serum	Muscle	Heart	Liver	Kidney	Brain
I	None	Av.	106·6	30·2	32·5	10·8	94·2	59·0	59·4	47·2	53·7
		S.E.	±1·3	±3·2	±3·1	±0·35	±3·0	±1·3	±4·1	±2·3	±2·0
II	NaH_2PO_4	Av.	100·9	26·5	29·8	10·8	90·8	59·6	61·2	53·6	56·5
		S.E.	±1·9	±3·6	±4·6	±0·47	±1·4	±1·5	±2·9	±2·5	±1·7
III	Me–Cl–Col–Ac	Av.	*97·1*	23·5	23·0	10·2	85·6	62·3	58·5	47·9	53·7
		S.E.	±1·8	±0·9	±3·0	±0·58	±3·1	±2·2	±3·7	±1·6	±1·5
IV	NaH_2PO_4 +Me–Cl–Col–Ac	Av.	*96·4*	34·0	31·9	9·6	*80·8*	*68·0*	58·4	*115·7*	55·3
		S.E.	±1·5	±4·6	±3·9	0·64	±3·2	±2·61	±3·2	±18·3	±1·3

Values in italics proved to be statistically significant ($P < 0·05$) as compared with the corresponding normal values (Group I).

unrelated to the question of specificity, it is of interest to mention that increases in the levels of phosphate (Table III) and Ca (Table II) observed in the kidney of the animals in group IV could well reflect the nephrocalcinosis already reported in connection with this electrolyte-steroid-cardiopathy (Selye and Mishra, 1958). Calcium variation in cardiac necrosis is probably not specific of that lesion, as it increased in both heart and muscle of group IV and no necrosis was ever detected in the muscle of that group. It is true that often a general dystrophic calcification will follow focal necrosis (Barr, 1932). However, Mg seems to be associated more specifically with heart necrosis of this type. An important decrease of that electrolyte (48 per cent) was seen in the necrotic heart of group IV, whereas no muscle Mg variation was observed in the same group (Table II). It is of interest to compare this drop of Mg in the present infarctoid lesion with the results of Tufts and Greenberg (1938) and Watchorn and McCance (1937) who could not alter the concentration of Mg in the heart, even though plasma Mg fell to half of the normal value in their conditions. Recently, MacIntyre and Davidson (1958) have shown that Mg deficiency can cause secondary K depletion, Na retention and hypercalcaemia in rats and they suggested that these secondary phenomena might be due to an effect of Mg deficiency on mitochondria. This finding suggests that Mg plays a role of primary importance amongst electrolytes in relation to metabolism of living tissues.

It is of interest to discuss hypochloraemia which was observed in Groups III and IV (Table III). Although some cardiac diseases can be produced after chronic Cl depletion (Winkler and Crankshaw, 1938), it is not yet known whether this condition is the primary causative factor or not.

It has been emphasised by many workers (Moulton, 1923 ; Darrow, Harrison and Taffel, 1939 ; Cotlove, Holliday, Schwartz and Wallace, 1951) that electrolyte analyses are less variable when expressed in terms of dry fat-free bases. The authors intend to report on that basis in future work in order to express the precise relationship of Mg to changes in water and lipid content of tissues. In conclusion, it is felt that the evidence presented in this study suggests that a Mg decrease is specifically involved in the mechanism of production of our experimental electrolyte-steroid infarctoid lesion. It is probable that the Mg disbalance affects the Mg-dependent enzymes in the heart. This possibility is under investigation at present.

SUMMARY

The tissue levels of 6 electrolytes were determined, in connection with an infarctoid heart lesion experimentally induced by the administration of NaH_2PO_4 and Me–Cl–Col–Ac in the rat. A marked decrease of Mg and an increase of Na and Ca were observed in the heart in which necrosis was produced by treatment with NaH_2PO_4 and Me–Cl–Col–Ac. The increase of Na and Ca in the necrotic heart was accompanied by an increase of these ions in the abdominal muscle. Hypokalaemia and hypochloraemia were also found in the phosphate-steroid-treated group.

In spite of the fact that comparatively large amounts of phosphate were administered, its content did not increase in the serum or abdominal muscle of the animals, although an increase was observed in the heart.

Na, K and Ca do not seem to have as much bearing on this heart necrosis as does Mg. The reasons for this hypothesis are discussed.

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THE USE OF ACQUIRED IMMUNOLOGICAL TOLERANCE AND IMMUNOLOGICAL "PARALYSIS" IN THE STUDY OF THE ANTIGENIC RELATIONSHIPS OF NORMAL AND ABNORMAL SERUM GLOBULINS

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THE abnormal globulins of myeloma, cryoglobulinaemia and macroglobulinaemia have been generally regarded as entities not present in normal serum. Evidence in favour of this hypothesis includes the wide range of physical properties encountered amongst these abnormal globulins and the specificity of their antisera after absorption with normal human serum to remove cross-reacting antibodies. Recently this view has been questioned by Deutsch, Morton and Kratochvil (1956) who by using a precipitin-inhibition technique, found that antibodies to a number of purified abnormal globulins could be completely combined by reaction with normal human serum globulin fractions. They concluded that the abnormal globulins were probably gross elevations of minutely occurring normal globulins. This conclusion has been criticised by Kabat (1957) on the grounds that the presence of the other normal globulin components which cross-react with the abnormal globulin might be sufficient to inhibit precipitation.

This paper describes the results of a fresh approach to this problem, using the phenomena of acquired immunological tolerance and immunological "paralysis" in rabbits. In the acquired immunological tolerance experiments rabbits were injected with relatively large doses of normal human plasma shortly after birth and attempts were made to elicit an antibody response to abnormal serum globulins in later life.

In the immunological "paralysis" experiments adult rabbits were given massive injections daily of normal human plasma for periods of 2–3 weeks. Under these conditions antibody production to the main components of plasma is suppressed, but detectable antibody is produced to minor components (Dixon and Maurer, 1955). Attempts were made to find specific antibodies to abnormal serum globulins in the sera of the infused rabbits.

MATERIALS AND METHODS

Normal human plasma.—Pools of oxalated plasma were prepared with at least 5 donors in each pool, and stored at −15°.

Concentrated plasma.—This was prepared by dialysing aliquots of the pooled plasma against 15 per cent dextran solution. The protein concentration of the final product was approximately 11g. per cent.

Pathological sera.—Serum was obtained from 5 myeloma patients attending hospital in Melbourne. The relevant characteristics of the sera are given in Table I. Serum was also obtained from 2 patients with macroglobulinaemia (Curtain, 1959) and a patient with cryoglobulinaemia (Barnett, Curtain and Hayes, 1956).

Bence-Jones protein.—Three of the myeloma patients excreted large quantities of Bence-Jones protein. The protein was precipitated in each case by half-saturating the urine with ammonium sulphate. The precipitate was recovered by centrifugation, washed twice with a 50 per cent ammonium sulphate solution, dissolved in distilled water approximately twice the volume of the original urine and reprecipitated by half-saturation with ammonium sulphate. The precipitate was dissolved in a minimum volume of distilled water, dialysed for 48 hr. at 2° against 4 changes of distilled water and then Seitz filtered. The final solutions contained 2–3 g./100 ml. of protein. It is believed that this method of preparation did not denature the proteins. All 3 preparations gave a single peak on moving boundary electrophoresis in pH 8·6, I = 0·1, sodium veronal buffer and a single sharp line in the Ouchterlony plate

Abnormal serum globulins.—The macroglobulins were isolated as described by Curtain (1959). The cryoglobulin was isolated as described by Barnett, Curtain and Hayes (1956). The myeloma globulins were isolated by starch block electrophoresis (Kunkel, 1954).

Rabbits.—The rabbits used in the experiments were obtained from the hybrid stock which has been interbred (with the occasional introduction of new breeding bucks) at the Baker Institute for 20 years.

Antigen-antibody titrations.—Antibody titres were obtained either by the ring test (Fornet and Müller, 1910) or by the sensitive tanned-sheep cell technique (McKenna, 1957).

Antigenic specificity was studied by the technique of double diffusion in agar (Ouchterlony, 1953).

TABLE I.—*Abnormal Sera Used in the Acquired Immunological Tolerance Experiments*

Name	Concentration of abnormal globulin in serum (g./100 ml.)	Mobility of abnormal globulin in pH 8·6, I = 0·1 veronal buffer ($\times 10^{-5}$ cm^2 v^{-1} sec^{-1})	Excretion of Bence-Jones protein in urine (g./day)
C. J—	8·7	0·97	—
J. B—	11·5	1·37	27
D. M—	9·6	1·08	33
L. C—	10·7	1·27	24
E. A—	8·3	2·65	—

RESULTS

Studies on the Specificity of Acquired Immunological Tolerance

The use of acquired immunological tolerance to study the antigenic relationship of normal and abnormal serum globulins must depend on whether tolerance can be proven to be specific for closely related antigens.

Most of our knowledge of the specificity of acquired immunological tolerance has been based on experiments involving heterologous, or native and modified, protein antigens (Hanan and Oyama, 1954; Cinader and Dubert, 1955, 1957). The abnormal serum globulin and the Bence-Jones protein of myeloma from the same patient represent a pair of immunologically individual but cross-reacting autologous antigens (Korngold and Lipari, 1956) and their behaviour in an immunological tolerance experiment should enable us to define within much closer limits than before the specificity of the phenomenon.

The antigenic relationship of 3 pairs of myeloma globulins and Bence-Jones proteins, from patients J. B—, D. M— and L. C—, are shown in Fig. 1. In each case the spur on the myeloma globulin line indicates that the Bence-Jones protein has fewer antigenic determinants than its corresponding serum globulin.

Five rabbits from each of 3 litters (0–6 hr. old) were each injected intraperitoneally with 0·5 ml. of a 2 per cent solution of one of the Bence-Jones proteins. The remaining rabbits in each litter were reserved as controls. At 65, 75 and

85 days after birth each rabbit was injected intramuscularly with 30 mg. of Bence-Jones protein in Freund's adjuvant. The controls showed a normal antibody response as measured by the ring test. Titres in all cases exceeded 1/1024. With 2 exceptions none of the rabbits injected at birth gave any detectable antibody response, even when their sera were tested by the tanned sheep-cell technique. At 95, 102 and 109 days the unresponsive rabbits were challenged by intramuscular injections of 30 mg. of the myeloma globulin in Freund's adjuvant corresponding to the Bence-Jones protein which they had received at birth. At 122 days the rabbits were bled and the sera examined on the Ouchterlony plate. A mixed response was obtained. Three of the rabbits failed to produce antibody at all ; 8 produced antibody only to the antigenic determinants of the myeloma globulin which were not present in the Bence-Jones protein, and 4 produced antibody to all the antigenic determinants present in the myeloma globulin *i.e.* the immunological tolerance had broken down.

This variable response on the part of immunologically tolerant animals to cross reacting antigens appears in the results of Cinader and Dubert (1955, 1957) who studied bovine serum albumin and its azo-derivative.

Effect of age of challenge with myeloma globulin

It was thought that the age at which the rabbits were challenged might be of importance in determining the nature of the response to the cross-reacting antigen. To test this hypothesis the above experiment was repeated, varying the age of challenge with the myeloma globulins. Three groups of 8 rabbits (0–6 hr. old) were each injected with 0·5 ml. of a 2 per cent solution of one of the Bence-Jones proteins. Four rabbits in each group were injected with the Bence-Jones protein as before at 35, 42 and 49 days. None of the rabbits gave a detectable antibody response. They were then injected with the corresponding myeloma globulin at 56, 63 and 70 days. They were bled at 82 days and the sera examined on the Ouchterlony plate. Only one rabbit formed antibody to the antigenic determinants of the Bence-Jones protein. The others formed antibodies only to those determinants which the myeloma globulins did not have in common with the Bence-Jones proteins. The other 4 rabbits in each group were injected with the Bence-Jones proteins in Freund's adjuvant at 95, 102 and 109 days. Three of the 12 rabbits (one in each group) produced antibody to the Bence-Jones proteins. The unresponsive rabbits were then injected with the appropriate myeloma globulins at 116, 123 and 130 days. They were bled at 143 days and the sera examined on the Ouchterlony plate. It was found that immunological tolerance of the majority of the animals in this experiment had broken down.

Those animals which had reacted to only the antigenic determinants of the myeloma globulins were injected with the globulins in Freund's adjuvant at 160, 175 and 190 days. The rabbits were bled at 200 days and it was found in all cases that their sera reacted with the Bence-Jones proteins to a titre of 1/1024 by the ring test.

Duration of tolerance to a single antigen

Eight rabbits from 2 litters were injected at 0–6 hr. with 0·5 ml. of 2 per cent Bence-Jones protein (" J. B ") solution. Four of the rabbits were given injections

of 20 mg. of the Bence-Jones protein in Freund's adjuvant at 10 day intervals from 40 days. At the time of writing (300 days) no antibodies have been detected in the sera of these rabbits. Two of the rabbits were given injections of 20 mg. of Bence-Jones protein in Freund's adjuvant at 10 day intervals from 80 days and the remaining 2 were injected with the same dose at 10 day intervals from 110 days. One of the 80 day rabbits started to produce antibodies at 97 days and attained a maximum titre of 1/2560 (ring test) by 150 days.

It was concluded from these experiments that the mechanism governing immunological tolerance in the rabbit was capable of distinguishing between a pair of autologous, cross-reacting proteins, provided that the second protein was injected at or before 56 days after birth.

Experiments with whole plasma

Immunological tolerance to the components of myeloma serum.

Five rabbits (0–6 hr. old) from each of 3 litters were injected intraperitoneally with 0·75 ml. each of the concentrated pooled human plasma. At 35, 42 and 49 days they were injected intramuscularly with 4 ml. of concentrated pooled plasma in Freund's adjuvant. At 55 days no antibodies could be detected in their sera. The 15 rabbits were then divided into 5 groups of 3 and each group was injected with 4 ml. (in Freund's adjuvant) of the serum from an individual myeloma patient at 55, 62 and 68 days. The rabbits were bled at 75, 80 and 90 days and their sera tested for antibodies by means of tanned sheep-cells coated with the appropriate myeloma protein. In no case was it possible to detect antibody to the myeloma protein.

All the control rabbits gave high titre antibodies (1/1024 by the ring test) to normal human plasma by the 55th day. In order to determine whether the tolerant rabbits could still form antibodies both these and the control rabbits were given injections of 25 mg. of bovine serum albumin (Armour) in Freund's adjuvant at 100, 107 and 114 days and were bled at 125 days. All of the animals gave high antibody titres (1/1024 by the ring test) and the sera of 2 of the previously tolerant rabbits contained weakly reacting antibodies (titres 1/128–1/256 by the ring test) to human serum albumin.

Immunological tolerance to sera from cryoglobulinaemia and macroglobulinaemia

These experiments were repeated using sera from the case of cryoglobulinaemia and the 2 cases of macroglobulinaemia in the place of the myeloma sera. Again, none of the rabbits produced antibodies to the abnormal globulins.

Experiments with "Immunological Paralysis"

Five 90 day old rabbits were given normal human plasma (10 ml. per kg.) by combined intravenous and subcutaneous routes (in a ratio of 1 : 3) daily for a period of 33 days. One of the rabbits died at 27 days, with a marked anaemia and the arteritis and endocarditis of serum sickness. The surviving rabbits were bled at 34 days and the sera tested for antibodies. The sera reacted weakly (titre 1/64) with human γ-globulin and not at all with human serum albumin (both Australian Commonwealth Serum Laboratories Special Products), in the ring test. All the sera reacted to give multiple bands in the Ouchterlony plate

(Fig. 2) with whole human serum, and a single fairly strong band with the abnormal globulins, with the exception of one myeloma globulin (E. A.). The lines in these plates were enhanced by the presence of 0·01 per cent cadmium nitrate in the agar (Crowle, 1958).

Presence of antibodies cross reacting with abnormal serum globulins in the sera

The 5 myeloma globulins, the cryoglobulin, the two macroglobulins and normal human γ-globulin were adsorbed to tanned sheep-cells. These cells were then tested against the sera from the 4 rabbits. The results are given in Table II.

TABLE II.—*Titres Obtained by the Tanned Sheep Cell Technique Against Various Antigens with the Sera of Rabbits Infused with Whole Normal Human Plasma. Titres Expressed as Reciprocal Dilutions*

Antigen	Rabbit Number 1	2	3	4
Myeloma globulin—				
C. J— . . .	8,000	10,000	7,000	9,000
J. B— . . .	12,000	16,000	10,000	12,000
D. M— . .	15,000	10,000	9,000	11,000
L. C— . . .	12,000	10,000	12,000	8,000
E. A— . . .	200	350	100	100
Macroglobulin A . .	9,000	5,000	5,000	3,000
Macroglobulin B .	800	1,600	2,000	200
Cryoglobulin . .	8,000	6,000	6,000	4,000
Normal γ-globulin .	850	540	600	300

Cells coated with one of the macroglobulins and the cryoglobulin gave 10 times the titre obtained with cells coated with normal γ-globulin against the sera from the 4 rabbits. Cells coated with the other macroglobulin gave a titre slightly less than that obtained with cells coated with γ-globulin against sera of 2 of the rabbits and twice that value with the remaining rabbits. Cells coated with the myeloma globulins, with one exception, gave high titres with the sera from all 4 rabbits. The exception, which was a β-globulin (E. A.) reacted weakly with the sera of all the rabbits.

DISCUSSION

The Specificity of Acquired Immunological Tolerance

In the case of rabbits made immunologically tolerant to a Bence-Jones protein by its injection shortly after birth, the age of challenge with a cross reacting myeloma protein appears to be the most important factor in determining whether antibodies will be produced only to those antigenic determinants which the myeloma globulin did not have in common with the Bence-Jones protein. Older rabbits challenged with myeloma globulin gave a variable response, suggesting that the diminution of the specificity with increasing age varied with individual rabbits.

Studies with abnormal sera

If we assume that the antigenic differences between the abnormal globulins and their cross-reacting normal serum components are no less than those between

a Bence-Jones protein and its corresponding myeloma globulin, then we may conclude from the immunological tolerance experiments that the abnormal sera examined contain the same antigenic determinants which are present in normal serum. The results of the immunological " paralysis " experiments suggest that many of these determinants are to be found amongst the minor components of normal human serum.

These conclusions agree with those of Deutsch, Morton and Kratochvil (1956) and Smith, Brown, McFadden, Buettner-Janusch and Jager (1956). However, it is difficult to reconcile their hypothesis, that the abnormal globulins are gross elevations of minutely occurring normal globulins, with the wide range of physical and chemical properties reported for the former in the literature. The reports of the many individually distinguishable proteins formed in different cases of multiple myeloma and macroglobulinaemia are documented in a recent review by Putnam (1957).

Unless we postulate the existence of a multiplicity of minor normal globulin components from which the abnormal globulins can arise some fresh hypothesis, such as that outlined below, is necessary to reconcile the diversity of the abnormal globulins with the results presented in this paper and the conclusions of Deutsch *et al.* (1956) and Smith *et al.* (1956).

A large number of proteins with differing physical and chemical properties could be created by varying combinations from a relatively small pool of sub-units. If we accept the template hypothesis of protein synthesis it is possible to imagine that in the normal cell the sub-units are assembled on, or under the influence of, the template to give a normal globulin molecule. In the pathological cell, however, part of this template has been substituted by part of a template from another cell. If the other cell belongs to a clone which produces a different normal globulin to the precursors of the pathological cell then the abnormal globulin would be a hybrid between 2 normal globulins.

This process of template recombination could either be a consequence or a cause of the proliferation of the pathological cell.

Direct proof of the hybrid nature of the abnormal globulins can only come from immunological and physico-chemical comparison of these proteins with isolated fractions from the globulin spectrum. Although the micro-heterogeneity

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EXPLANATION OF PLATE.

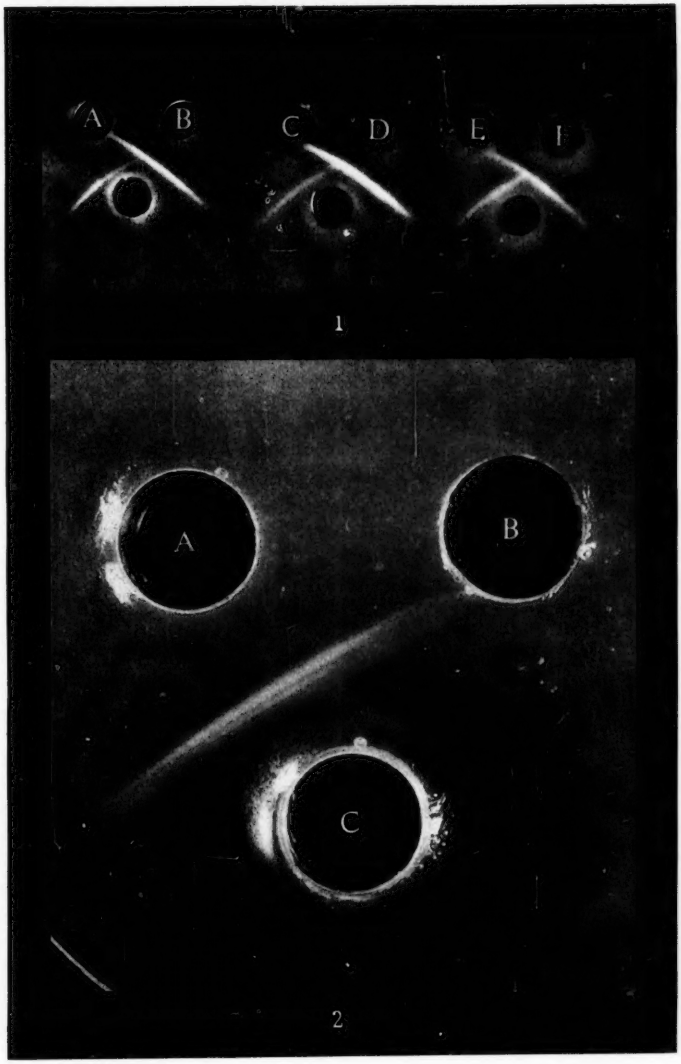
FIG. 1.—Ouchterlony print showing the immunological relationships between the myeloma globulins and Bence-Jones proteins used to study the specificity of acquired immunological tolerance.

A—Myeloma globulin " J. B."
B—Bence-Jones protein " J. B."
C—Myeloma globulin " D. M."
D—Bence-Jones protein " D. M."
E—Myeloma globulin " L. C."
F—Bence-Jones protein " L. C."

Each lower well contained the antiserum to the appropriate myeloma globulin.

FIG. 2.—Ouchterlony print of the reaction between the serum of a rabbit which had been infused with large amounts of normal human plasma (rabbit 2 of Table II) and whole normal human serum and myeloma globulin " D. M."

A—Myeloma globulin " D. M."
B—Whole normal human serum.
C—Rabbit serum.



Curtain.

of the serum globulins has been recognised for some time, only with the introduction of techniques of protein chromatography (Peterson and Sober, 1956; Porter, 1955; Tiselius, Hjerten, and Levin, 1956) has the possibility of separating quantities of the minor globulin components been considered. Future studies could involve the combination of these techniques with those of immunology. The problem is an important one. To quote Putnam (1957), "If the myeloma globulins are regarded as normal in origin a mechanism must be devised to account for their selective synthesis. If regarded as abnormal or anarchic proteins the phenomenon resembles a mutation."

SUMMARY

Rabbits with an acquired immunological tolerance to a Bence-Jones protein were injected with 3 doses in Freund's adjuvant of an antigenically related myeloma globulin from the same individual.

Nearly all those rabbits which were less than 56 days old on injection formed antibodies to the antigenic determinants which the myeloma globulin did not have in common with the Bence-Jones protein. No antibodies were formed to the Bence-Jones protein itself.

Rabbits 55 days-old, with an acquired immunological tolerance to normal human plasma were injected with sera in Freund's adjuvant, from cases of myeloma, macroglobulinaemia, and cryoglobulinaemia. In no case was it possible to detect antibodies to either normal or abnormal human serum in the sera of these rabbits.

A state of immunological unresponsiveness to the major components of normal human plasma was induced in adult rabbits by the daily injection of large doses of plasma. The sera of these rabbits reacted weakly with normal human serum, but quite strongly with many abnormal serum globulins.

From these experiments it was concluded:

(*a*) That, under certain conditions the mechanism governing acquired immunological tolerance is sensitive to relatively small differences between cross-reacting antigens.

(*b*) That, assuming that the abnormal serum globulins are no more closely related to the normal globulins than the myeloma globulins are to the Bence-Jones protein, the experiments with whole plasma indicate that the sera of the cases of myeloma, macroglobulinaemia and cryoglobulinaemia studied contain only the antigenic determinants which are present in normal serum.

(*c*) That those parts of the abnormal globulin molecules which do not cross react with the major globulin fractions are related to the minor components of normal human serum. However, it is felt that the physical and chemical properties of abnormal globulins vary too widely to be merely gross elevations of the minor normally occurring serum globulins, unless we postulate the occurrence of a very large number of the latter. It is suggested, as an alternative hypothesis, that the abnormal globulins are hybrids, formed from two or more normal serum globulins.

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THE RELATIONSHIP OF TISSUE FORMATION AND DESTRUCTION TO THE LEVELS OF THE SERUM PROTEIN-BOUND CARBOHYDRATE IN RATS BEARING THE WALKER 256 CARCINOMA

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A RAISED level of total protein-bound carbohydrate in the serum is well recognised to occur in various forms of human carcinoma. This elevation has tentatively been ascribed to tissue destruction on the one hand (Seibert, Seibert, Atno and Campbell, 1947) and to proliferation of tissue on the other (Shetlar, Foster, Kelly, Shetlar, Bryan and Everett, 1949). Shetlar (1952) believed that tissue proliferation probably accounted for the raised albumin-bound polysaccharide and that the elevated α-globulin polysaccharide might perhaps be related to tissue destruction. The changes in serum polysaccharide levels have also been followed in animals bearing transplantable tumours. Catchpole (1950), employing fibrosarcomata in two strains of mice, found enhanced amounts of plasma glycoproteins in the presence of large tumours, but the release of carbohydrate was not always proportionate to the size of the tumour. Catchpole also noted a distinct rise of the water-soluble glycoproteins in the connective tissue abutting on the tumour. He thought that the increased circulating glycoprotein arose from depolymerization of the ground substance of the connective tissue at the site of invasive growth with subsequent absorption of the smaller, water-soluble and diffusible glycoprotein fractions. An increased concentration of serum protein-bound carbohydrate (nonglucosamine) occurred in rats bearing the Walker 256 carcinoma and it was suggested that the rise, which was particularly associated with the albumin fraction, might be related to the growth of the tumour (Shetlar, Erwin and Everett, 1950). Weimer, Quinn, Redlich-Moshin and Nishihara (1957) observed an increase in the mucoprotein concentration but a decline in the other serum glycoproteins of rats implanted with the Walker carcinoma. These authors considered that the increased mucoprotein fraction might represent a systemic response to tissue damage whilst the decreases in the other glycoproteins might be due to their utilization by the rapidly growing tumour. The serum carbohydrate levels have been followed in rats bearing a transplanted sarcoma by Baldwin and Harries (1958), who found the rise in total glycoprotein to be largely a reflection of changes in the mucoprotein fraction. Baldwin and Harries attributed the abnormal production of serum mucoprotein to processes occurring during tissue proliferation rather than to elimination of tissue glycoproteins in consequence of degenerative changes in the tumour and surrounding tissue, but in this interpretation only the weight of the whole tumour was considered.

Diverse opinions thus exist as to which fractions of the serum proteins carry raised amounts of polysaccharide both in human and in experimental neoplasia.

Furthermore, agreement has not been achieved on the parts played by tissue proliferation and tissue destruction in the elevation of the serum protein-bound carbohydrate levels. Differences in technical methods may possibly account for some of these divergences but another explanation may be found in the fact that in none of the above experimental studies were the relative proportions of viable and necrotic tumour tissue assessed, the state of the tumours being judged solely by their size. In the present investigation the serum protein-bound carbohydrate concentrations were estimated in rats bearing the Walker 256 carcinoma and account was taken of the differing amounts of dead and live tissue that co-exist in individual tumours. The reaction of the connective tissue surrounding the tumours was also noted histologically. Increases were found in the hexose bound by the serum total protein, by the α_2-globulin and by the mucoprotein. It appears that these increases depend on the reaction induced by the tumour in the surrounding tissues rather than on the amount of living or dead tumour. In an attempt to isolate the effects of the necrotic element in the Walker carcinoma, the polysaccharide levels were estimated before and after treatment of tumour-bearing rats with a powerful inhibitor.

METHODS

Initial studies employed the Brown-Pearce carcinoma implanted into the testes of rabbits but this method had to be abandoned because many of the tumours regressed completely after attaining only a moderate size and the changes in serum carbohydrate levels were insignificant, even when the tumour had metastasised.

These difficulties were overcome by using the Walker 256 carcinoma in rats. The tumour was maintained by serial passage every 14 days and transplanted into experimental groups as required. Using aseptic precautions, viable-looking tumour was implanted subcutaneously by trocar and canula under ether anaesthesia into male Wistar rats, approximately 6 months old. Throughout the experiments the site of implantation was constantly the right flank and the size of the tumour implant was similar in each rat.

Blood was obtained from normal and from tumour-bearing rats at intervals of 7–30 days after implantation by cutting their throats under ether anaesthesia. At the same time haemoglobin estimation (alkaline haematin method) was made on some animals both normal and with tumours of different sizes. The rats were fasted for several hours before death.

Established Walker carcinoma undergoes complete regression in rats given L-*p*-di-(2-chloroethyl) aminophenylalanine (compound CB 3025) intraperitoneally (Bergel and Stock, 1953). Six tumour-bearing rats received CB 3025 in arachis oil by intraperitoneal injection, each animal having two doses each at the rate of 4 mg./kg. body weight with an interval of 3 days between doses. Blood for chemical analysis was obtained by cardiac puncture under anaesthesia before treatment as well as at the conclusion of the experiment.

Estimation of the relative amounts of viable and necrotic tumour

The Walker carcinoma, when growing subcutaneously, usually forms a well-defined tumour and, even when large, invasion of surrounding tissues is exceptional. In the main experiment, tumours of various sizes were carefully and cleanly excised, weighed and fixed in formol-saline. Each tumour-bearing rat had been weighed immediately before death so that, by difference, the weight of the carcase (*i.e.* the "weight of rat") was also known. Three to five blocks were taken parallel to one another through different levels of the whole tumour and paraffin sections were stained with haematoxylin and eosin.

In the stained sections it was possible to define with reasonable accuracy the boundaries of viable and necrotic tumour tissue, the former taking up more haematoxylin and the latter more eosin. With the aid of a photographic enlarger, a coloured image of the section at 6 magnifications was then used to obtain an outline diagram of the whole tumour section together with its viable and necrotic portions. The distinction between these parts was

checked microscopically. From the drawing the areas of the living and dead tumour tissue were next measured by planimeter. The average of the areas of necrotic and viable tissue in all sections from one tumour was used to calculate the ratio between the two parts. This ratio was taken to apply to the whole lesion and, though based on area in the first place, it was used to derive the following ratios by weight: weight of tumour to weight of rat, weight of necrotic tissue to weight of rat and weight of viable tissue to weight of rat.

Analytical procedures

The methods employed were those previously described (Heppleston and Keyser, 1957). In addition the serum proteins were fractionated by electrophoresis on paper strips, 10 cm. wide, which were divided longitudinally. Half of the strip was stained for protein with lissamine green, the bands being cut out and the dye eluted with barbitone buffer of pH 6·0. The optical density of the resulting solutions was measured photoelectrically, and from a calibration curve relating dye concentration to colorimeter readings the relative amounts of dye bound by the various protein fractions were calculated. From the other half of the paper strip the distribution of the protein-bound carbohydrate was estimated by the periodic acid-Schiff procedure (Köiw and Grönwall, 1952), the paper being scanned photoelectrically in the dry state, as suggested by Björnesjö (1955). Hexosamine was estimated by the method of Elson and Morgan (1933) after hydrolysis with N hydrochloric acid (Rimington, 1940), but maximum yields were only obtained after hydrolysis for 8 hr. In most instances mucoprotein was estimated as hexose (Winzler, Devor, Mehl and Smyth, 1948) but sometimes also as " tyrosine " using the Folin-Ciocalteu reagent.

RESULTS

The great majority of the implants were successful, tumour growth being apparent to palpation after approximately one week. Subsequently, however, the rate at which tumours enlarged was variable even though the size of the viable implant and the strain of the host were made as uniform as possible. A regular time-growth relationship, such as other authors claim, could not therefore be demonstrated. A few tumours, after growing for 2–3 weeks and reaching a size of 2–4 cm. across, underwent spontaneous regression and no tumour tissue or only necrotic material was evident at necropsy.

In attempting to measure the intensity of the stimulus provided by the tumour as a whole and by its viable and necrotic elements, their respective amounts have been expressed in relation to the carcase weight of the host animal. The weight of the whole tumour was obtained directly, but the weights of the viable and necrotic parts could only be derived indirectly and they must therefore be regarded as approximations. Necrotic Walker carcinoma often undergoes central liquefaction, but some of the fluid may be derived from the blood. In computing the area of necrotic tumour the liquefied zone had to be included, even though the extent of the necrotic element may have been exaggerated as a result. The absolute weights of the whole tumours, when arranged in a descending series, corresponded fairly closely with the ratios of tumour weight to rat weight, but correspondence was less evident between the estimated weights of viable and necrotic tumour and their respective ratios to the rat weight. The ranges of these ratios are given in Table I. Of the 31 rats, 25 had tumours weighing over 4 g. whilst 18 tumours were over 10 g. and 13 over 20 g. in weight.

TABLE I.—*Ranges of the Three Weight Ratios* (31 *Rats*)

	Whole tumour : rat	Viable tumour : rat	Necrotic tumour : rat
Minimum	1 : 429	1 : 1420	1 : 735
Maximum	1 : 2·7	1 : 9	1 : 3·7

The Figure shows typical electrophoresis curves for serum proteins and protein-bound carbohydrates, whilst Table II records the serum levels in the normal and all the experimental rats, irrespective of the size of their tumours. The number of animals in each category of Table II is not constant, because the amount of serum from a particular rat was sometimes insufficient for every

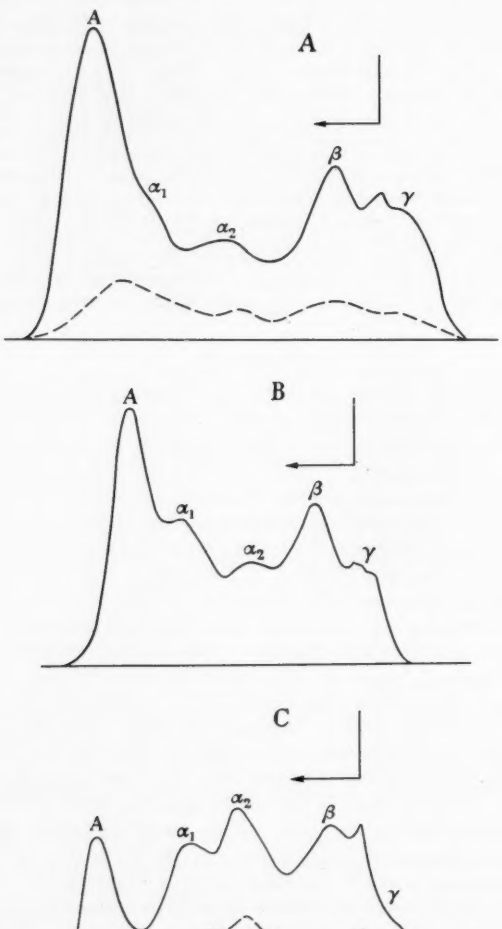

FIGURE.—Examples of the electrophoretic patterns of serum proteins of (A) normal and (B and C) tumour-bearing rats, obtained by passing the paper strips through an E.E.L. photoelectric " Scanner ". The unbroken line indicates protein (lissamine green staining) and the broken line carbohydrate (PAS staining). The nomenclature is arbitrary. Slight variations were sometimes found, *e.g.* two smaller peaks in the " α_2-globulin " position.

analysis. It must be emphasized that the nomenclature of these protein fractions is arbitrarily based on their sequence and not on relative mobilities. The assumption is made that no fraction has disappeared in the tumour-bearing rats and that no additional fractions have been formed. Albumin and α_1-globulin are not well separated in the normal rat nor are their hexose components but the distinction is clearer in the experimental animals. In these it appears that albumin concentration decreases and that the α_1-globulin increases, the two fractions tending to separate. Although α_1-globulin-bound hexose evidently rises, changes in albumin-bound hexose cannot be measured. It was thus impossible to assess the individual changes in these components statistically, and, because of the independent variation, great reliance cannot be placed on the average combined concentrations either of albumin plus $\alpha_1$globulin or of their carbohydrate fractions. Separation of β- and γ-globulin-bound hexose was sometimes too poor for individual measurement, but even where distinction was possible it seemed insufficient to justify statistical analysis. The average amount of β-globulin hexose is probably increased but the γ-globulin hexose concentration appears unaltered.

In the experimental group only the total serum protein and γ-globulin levels are diminished. The haemoglobin concentration, though not estimated in all rats, was reduced in tumour-bearing animals, the fall tending to be greatest in rats with the largest tumours and reaching as low as 6·7 g. per 100 ml. (average normal 14·8). However, depression of the haemoglobin level did not always occur, normal or only slightly reduced values sometimes being associated with large tumours. It is therefore difficult to assess the part played by haemodilution in the reduction of serum total protein and γ-globulin levels. If haemodilution occurred in tumour-bearing rats it was clearly inadequate to render insignificant the rises in concentration of the majority of serum constituents. For instance. the total protein-bound carbohydrate, whether expressed as absolute amount or as a percentage of the total protein, showed a highly significant rise despite an equally significant fall in total protein concentration. In a trial experiment, the concentration of serum hexosamine was found to be significantly raised in tumour-bearing rats and to run parallel with the carbohydrate elevation estimated as hexose (Table II). It was not therefore considered necessary to perform hexosamine estimations routinely.

Before attempting to interpret the changes affecting serum constituents in relation to the tumour mass and its living and dead components, it was necessary to eliminate the possible complication of bacterial infection in the tumour, a precaution to which previous investigators do not refer. Table II therefore includes the serum changes in a sample series of rats whose tumours were sterile on aerobic and anaerobic culture. It was undesirable to culture all tumours, since some loss of tissue was thereby entailed and this might have affected the estimation of viable and necrotic tumour weights. The changes in the " sterile " group are somewhat less pronounced than those in the experimental group as a whole, but they were nevertheless statistically significant with one unexplained exception. Despite the discrepancy in regard to the absolute amount of total protein-minus mucoprotein-bound hexose, there is good agreement when this value is considered as a percentage of total protein. Moreover, on histological examination, none of the tumours used in the analysis showed obvious evidence of infection. It is therefore reasonable to conclude that infection was not a

TABLE II.—*Changes in the Serum Components of Tumour-bearing Rats*

Animal groups	Protein: Total g./100 ml.	Protein: (Per cent total protein) Albumin + α_1-globulin	α_2-globulin	β-globulin	γ-globulin	Total protein-bound hexosamine (per cent total protein)	Protein-bound hexose: Total Mg./100 ml.	Total Per cent total protein	Albumin + α_1-globulin Mg./100 ml.	Albumin + α_1-globulin Per cent total hexose
Normal rats	6·53 ±0·50 (15)	62·5 (9)	8·3 ±1·58 (9)	17·93 ±1·08 (9)	11·4 ±1·34 (9)	1·59 ±0·16 (6)	168·0 ±18·95 (15)	2·57 ±0·14 (15)	102 (8)	57·7 (8)
All tumour-bearing rats	4·96 ±0·75 * (56)	56·1 (35)	13·73 ±6·63 * (35)	22·96 ±3·7 * (31)	9·4 ±2·7 † (32)	2·20 ±0·38 † (6)	198·3 ±30·04 * (56)	4·06 ±0·69 * (56)	96 (41)	47·2 (41)
Rats with sterile tumours	4·84 ±0·68 * (13)	—	9·88 ±0·98 ‡ (9)	—	—	—	—	3·90 ±0·53 * (13)	—	—

Animal groups	Protein-bound hexose: α_2-globulin Mg./100 ml.	α_2-globulin Per cent total hexose	β-globulin Mg./100 ml.	β-globulin Per cent total hexose	γ-globulin Mg./100 ml.	γ-globulin Per cent total hexose	Mucoprotein Mg./100 ml.	Total minus mucoprotein Mg./100 ml.	Total minus mucoprotein Per cent total protein
Normal rats	22·2 ±6·27 (8)	12·6 ±3·18 (8)	37 (8)	21·0 (8)	15 (8)	8·8 (8)	8·25 ±1·12 (14)	152·7 ±13·54 (10)	2·40 ±0·14 (10)
All tumour-bearing rats	36·26 ±17·06 * (39)	17·66 ±8·11 † (39)	54 (29)	26·3 (29)	18 (29)	8·9 (29)	31·7 ±13·51 * (53)	166·5 ±22·92 † (53)	3·37 ±0·50 * (53)
Rats with sterile tumours	29·44 ±5·61 ‖ (9)	15·56 ±1·98 ‖ (9)	—	—	—	—	31·5 ±7·52 * (12)	154·2 ±24·9 (12)	3·23 ±0·46 * (12)

The values given are the means, with their standard deviations where possible.
Figures in brackets indicate the number of rats in each category.
p values . * $< 0·001$, † $< 0·01$, ‡ $< 0·02$, ‖ $< 0·05$.

complication and that the observed serum changes are attributable to the presence of tumours as such.

Statistical correlations with the ages of the tumours and their three weight ratios were calculated only in the cases of serum proteins showing significant changes in their carbohydrate fractions (Table III). No relationship exists between any of the serum changes and the age of the tumours. Depression of total serum protein (TP) is significantly correlated with the amounts of both whole tumour and its viable portion ($p = 0·01$) and in lesser degree with the quantity of necrotic tumour ($p = 0·05$). The raised percentage of α_2-globulin in the total protein (α_2 per cent) can only be related to the size of tumour as a whole when all rats are included ($p = 0·02$), but, if weight ratios below 1 : 200

TABLE III.—*Correlation of Serum Changes with the State of the Tumours*

	Correlation with							
	Age of tumour		Ratio weight of whole tumour to weight of rat		Ratio weight of viable tumour to weight of rat		Ratio weight of necrotic tumour to weight of rat	
Serum component	No. of rats	*p* value	No. of rats	*p* value	No. of rats	*p* value	No. of rats	*p* value
Total serum protein (g./100 ml.)	50	0·9	31	0·01	30	0·01	31	0·05
α_2-globulin (per cent of total protein)	35	0·8	19	0·02	19	0·1	19	0·1
	—	—	—	—	14	0·01*	16	0·05*
Total protein-bound hexose (per cent of total protein)	50	0·8	31	0·01	30	0·01	31	0·01
Total protein-bound hexose (mg./100 ml.)	—	—	31	0·4	30	0·3	31	0·2
	—	—	27	0·9*	23	0·5*	27	0·4*
α_2 - globulin - bound hexose (per cent of total hexose)	39	0·8	22	0·9	22	0·3	22	0·2
	—	—	—	—	16	0·05*	18	0·2*
α_2 - globulin - bound hexose (mg./100 ml.)	39	0·6	22	0·3	22	0·4	22	0·3
	—	—	18	0·5*	16	0·3*	18	0·3*
Mucoprotein-bound hexose (mg./100 ml.)	47	0·8	28	0·02	27	0·05	28	0·05
Total protein minus mucoprotein-bound hexose (per cent of total protein)	—	—	28	0·01	27	0·3	28	0·8
	—	—	—	—	20	0·02*	24	0·1*

* Indicates the *p* values when only ratios over 1 : 200 are included.
"Weight of rat" refers to the weight of the tumour-bearing animal minus the weight of the tumour.

are excluded a significant correlation exists with the amount of viable tumour ($p = 0{\cdot}01$) and less so with the necrotic element ($p = 0{\cdot}05$). Elevation of total protein-bound hexose as a percentage of total protein (TPH per cent) is correlated to the same degree with all 3 weight ratios ($p = 0{\cdot}01$), but this is probably a reflection of the diminution in TP, since the absolute amount of total protein-bound hexose (TPH) is not related to any of the three weight ratios and the TP depression is significantly related to all these ratios. The raised proportion of α_2-globulin-bound hexose in the TPH (α_2 H per cent) is not related to any of the weight ratios when all rats are considered, but there is a relatively weak correlation with the amount of viable tumour when only lesions with a ratio above 1 : 200 are included ($p = 0{\cdot}05$). The increased concentration of α_2-globulin-bound hexose (α_2H) bears no relation to the weight ratios. Elevation of mucoprotein-bound hexose concentration (MH) shows a fairly high correlation with the size of the whole tumour ($p = 0{\cdot}02$) and a less significant one with both the viable and the necrotic portions ($p = 0{\cdot}05$). The rise in level of total protein hexose minus mucoprotein hexose calculated as a percentage of total protein correlates with the weight of tumour as a whole ($p = 0{\cdot}01$) and less well with the amount of viable tumour if only lesions with a ratio over 1 : 200 are considered ($p = 0{\cdot}02$).

The series of tumour-bearing rats treated with compound CB 3025 is too small for statistical treatment of the results, but distinct trends are evident.

Disappearance of the whole tumour or of its viable portion was associated with a rise in TP and a fall in MH, thereby reversing the changes produced in rats by tumour alone. Treatment also reversed the rise in TPH per cent ; a tendency for the TPH to rise may well be a reflection of the increased concentration of TP.

DISCUSSION

The present results and previous observations all show a reduced concentration of serum total protein in animals bearing experimental tumours, but the changes in the level of the various protein-bound carbohydrate fractions do not exhibit so consistent a pattern in the different investigations. Recent evidence derived from rats with various types of tumour, and here confirmed, establishes that there is a rise in MH concentration (Weimer *et al.*, 1957 ; Baldwin and Harries, 1958). We also found an elevation of TPH, as did Shetlar *et al.* (1950) and Baldwin and Harries, but Weimer *et al.* failed to do so even though they used the Walker carcinoma in rats. However, these latter authors concur in finding the TPH per cent to be increased, thereby indicating a relative increase in the amount of carbohydrate bound to protein. No agreement yet exists on changes in the non-mucoprotein hexose concentration, the proportion in relation to TP being depressed (Weimer *et al.*) unchanged (Baldwin and Harries) or elevated (present results). We found the α_2 per cent to be significantly elevated and also the α_2H and α_2H per cent ; in this regard experimental neoplasia appears to resemble human malignant disease (Siebert *et al.*, 1947 ; Almquist and Lausing, 1957).

Hokkanen *et al.* (1956) observed an increase of serum mucoprotein (MP) in rats with an ascites tumour and they related the rise to the active phase of tumour development, though the maximum MP level was only reached after regressive changes became apparent histologically in the tumour. Although these authors estimated the proportion of necrotic cells in the ascitic fluid they made no such assessment on the solid tumour tissue. Hokkanen *et al.* also noted that the mucoprotein concentration of the ascitic fluid rose and fell in parallel with the serum changes, but the ascitic levels were always lower than those in the serum. This finding could be interpreted as evidence against the tumour's being the source of serum mucoprotein. Weimer *et al.* attempted to separate the viable and necrotic tissue in Walker 256 carcinomata on the basis of gross visual inspection, in order to estimate their respective total protein- and mucoprotein-bound carbohydrate contents. They found significant rises only in the necrotic tissue and thought that they were due to blood trapped in the tumour. These authors also noted that the levels of protein-bound carbohydrate in the tumour extracts were unaffected by the size of the tumour or its age and bore no relation to the serum concentrations. They therefore doubted whether the carcinoma itself was an important source of serum glycoproteins.

In trying to determine the reason for the elevation of serum protein-bound carbohydrates in experimental neoplasia, inadequate attention has, in the past, been devoted to the pathological features of the tumour. Our experiments were so designed that a statistical assessment could be made of the relationship between the serum changes and tissue formation and destruction, as judged by the amounts of living and dead tumour respectively. The results do not provide a clear distinction between the effects of viable and necrotic tumour (Table III). It is, however, important to note not only the lack of relationship between the

weight ratios and TPH elevation but also their lack of correlation with the rise in α_2H. Moreover, according to our observations, the increases of serum protein-bound carbohydrates were not always proportionate to the size of the tumour or of its viable and necrotic portions. These findings suggest that the cause or causes of the serum protein-bound carbohydrate changes may be connected with some property of the tumour we have not investigated or may even lie outside the tumour. Treatment of tumour-bearing rats with a powerful inhibitor reverses the major serum changes but does nothing to elucidate their causation. Attention was paid to the histological changes in the tumour and in the surrounding connective tissue. Necrotic tumour usually shows considerable polymorph infiltration. Immediately external to the zone of viable tissue, most of which is situated at the periphery of the tumour, inflammatory activity is usually well developed. In rats with totally necrotic tumours, irrespective of whether the necrosis occurred spontaneously or was induced by treatment with inhibitors, the surrounding inflammatory reaction becomes less active and fibrosis predominates, whilst the serum changes revert to normal. We have already shown that tissue formation, as judged by experimentally-induced inflammatory fibrosis in rabbits, is not related to serum protein-bound carbohydrate elevation but that this rise coincides with the acute phase of the inflammatory process (Heppleston and Keyser, 1957). In this connection it is worth recalling Catchpole's (1950) finding of increased water-soluble glycoproteins in the connective tissue around sarcomata in mice, but he made no histological observations. It must not necessarily be assumed from these observations that the inflammatory reaction around tumours liberates glycoproteins locally and that these are then absorbed to produce elevation of the serum levels. The increased tissue glycoprotein could equally be the result of inflammatory exudation of plasma that already has a high glycoprotein content. There is as yet insufficient information to be certain that the tissue and serum glycoproteins are chemically identical under experimental conditions.

SUMMARY

In an attempt to decide whether tissue formation or destruction is responsible for increased concentration of serum protein-bound carbohydrates, these concentrations were estimated in rats bearing the Walker 256 carcinoma and an assessment was made of the amounts of viable and necrotic tumour tissue present in relation to the size of the individual animal. Significant elevations occurred in the amounts of hexose bound by serum total protein, by α_2-globulin, by mucoprotein and by nonmucoprotein. These changes do not appear to depend on tissue formation or on tissue destruction as judged by the amount of viable or necrotic tumour respectively. Our results thus appear to dispose of the original question and to suggest instead that factors other than the amount of living or dead tissue may provide the stimulus for the changes in concentration of the serum protein-bound carbohydrates. In particular, the findings raise the possibility that the inflammatory reaction evoked by the tumour in the immediately adjacent connective tissues may be an essential element in the process leading to the serum changes.

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USE OF FOETAL HAEMOPOIETIC TISSUE TO PREVENT LATE DEATHS IN RABBIT RADIATION CHIMAERAS

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It is now well known that animals exposed to an expectedly lethal dose of X-rays will in many cases survive if they are injected intravenously with normal haemopoietic tissue (Lorenz, Uphoff, Reid and Shelton, 1951).

This beneficial effect depends upon successful transplantation and proliferation of the donor cells in the marrow and lymphoid tissue of a host whose immunological defences have been suppressed by the irradiation (Ford, Hamerton, Barnes and Loutit, 1956).

If treatment is by isologous tissue, that is from an animal of the same inbred strain, recovery is lasting and the life expectancy is little reduced, but if homologous or heterologous material is used recovery is but temporary and there is a high late mortality.

These delayed deaths constitute a serious drawback to any possible clinical application of marrow transplantation and although there seems to be general agreement that they are directly or indirectly the result of an antigen-antibody reaction, the exact mechanism is in dispute. Makinodan and his associates (1958) consider it a delayed immunological response on the part of recovered host cells to the foreign tissue, whilst Barnes, Ford, Ilbery, Koller and Loutit (1957) favour the idea that it is due to a reaction by the graft against the host. Recent evidence has given much support to this second theory (Trentin, 1957; Weyzen and Vos, 1957; Uphoff and Law, 1958; Porter and Murray, 1958*a*; Ilbery, Koller and Loutit, 1958).

If the hypothesis of a graft-versus-host reaction is correct then it would be reasonable to suppose that late deaths might be avoided by using immunologically immature and inactive foetal tissue instead of adult material to produce the chimaeras. Preliminary reports of work along these lines in mice appear encouraging (Barnes, Ilbery and Loutit, 1958; Uphoff, 1958).

In the present paper it will be shown that late deaths in irradiated rabbits can be prevented if each animal is treated with a suspension of haemopoietic cells from the liver of a 20-day or a 27-day-old rabbit foetus instead of with adult bone marrow. Treatment with neonatal liver is less effective.

MATERIALS AND METHODS

Animals.—Young adult Chinchilla rabbits, which were not inbred in the genetic sense, weighing 2–3 kg. were used throughout.

X-irradiation.—Total body X-irradiation was administered as a horizontal beam to 2 animals at a time from a Westinghouse machine under the following conditions: 220 kV., 12·5 mA., 70 cm. target to skin distance, 1·0 mm. Cu and 1·0 mm. Al filters, H.V.L. 1·8 mm. Cu,

dose rate to skin 33·4 r per minute, to centre of animal 21·1 r per minute. The dosage was divided; an initial dose of 600 r at the centre of the animal was followed 24 hours later by 500 r and after another 24 hours by a further 500 r, giving a total of 1600 r (LD_{100} 30 days).

Adult bone marrow.—Marrow (Fig. 1) was collected under sterile conditions from the bones of all four limbs of female rabbits as previously described (Porter and Murray, 1958*a*).

Foetal and neonatal liver.—At the 20th day and the 27th day of pregnancy (normal period of gestation in the rabbit is 31 days), rabbits were killed by an overdose of Nembutal and the foetuses taken out of the uterus aseptically. Each foetal liver (Fig. 2) was quickly removed, suspended gently in 10 ml. of normal saline, and filtered through nylon mesh of 90 μ porosity. Injections of these cell suspensions were given into the marginal ear vein of the recipient rabbit 1–3 hr. following the last dose of irradiation and within 1 hr. of the death of the mother rabbit. Suspensions of neonatal (day of or day after birth) rabbit liver were prepared in a similar fashion. Controls each received 10 ml. of saline intravenously. The gonads from each foetus were fixed and later examined microscopically to determine the sex of the donor.

Antibiotic therapy.—All rabbits were given tetracycline hydrochloride intramuscularly 50 mg. per day for the first 2 weeks following irradiation.

Haematology.—Total and differential leucocyte counts, platelet counts, microhaematocrits and haemoglobin estimations were determined every 2 days post-irradiation for the first 30 days and once per week subsequently. Blood films were examined at the same time intervals and female sex chromatin sought in the heterophil leucocytes (sometimes called amphophils or pseudo-eosinophils) which correspond to human neutrophils (Porter, 1957).

The rabbits were weighed daily. Necropsy was performed on all animals that died.

Experimental procedure

A total of 135 male Chinchilla rabbits were exposed to 1600 r whole body irradiation given in divided dosage. Of these 5 (3·7 per cent) died in shock shortly after the first dose of X-rays. The surviving animals were divided into 5 groups (Table I).

RESULTS

Survival

Table I shows that all 20 rabbits receiving X-irradiation alone (Group I) were dead by the 13th day, the mean survival time being 8·1 days. Of the 110 rabbits similarly irradiated but subsequently treated with various types of homologous haemopoietic tissue intravenously, many showed good recovery from the early radiation effects and the percentage survival at 3 weeks was approximately the same for all groups (50–60 per cent). In the case of the rabbits given

TABLE I.—*Effect of Homologous Haemopoietic Tissue on Survival of X-irradiated Male Rabbits* (1600 *r in Divided Dosage*)

Group	Number of rabbits	Haemopoietic tissue used in treatment	Mean number of nucleated cells injected ($\times 10^6$)	Percentage of animals showing initially successful marrow transplant	Percent survivors after weeks						
					2	3	4	6	8	13	17
I	20	None	—	—	0	0	0	0	0	0	0
II	20	Adult bone marrow	1,200	95	75	60	45	30	25	25	25
III	20	Newborn liver	500	65	60	50	40	30	30	30	30
IV	30	27-day foetal liver	1,000	90	67	60	50	50	50	50	50
VA	20	Liver from one 20-day foetus	350	75	60	55	45	45	45	45	45
VB	20	Liver from two 20-day foetuses	670	80	60	55	55	55	55	55	55

adult bone marrow (Group II) and new-born liver (Group III), there followed a marked reduction in the percentage survival at 8 weeks to 25 and 30 per cent respectively. This was due to the death of many animals from so-called " secondary disease " (Congdon and Urso, 1957) in which progressive, severe wasting began at the 2nd–6th weeks, followed by diarrhoea, a leucocytosis and often terminal jaundice. The survival pattern of those receiving foetal liver (Groups IV, V*a* and V*b*) was, however, quite different far fewer animals dying between the 3rd and the 8th weeks after treatment. Table II shows that this improvement in late survival was accounted for by the elimination of " secondary disease " as a cause of death.

Success of haemopoietic transplant

Successful homotransplantation was assumed if female type heterophils appeared in blood smears taken from the irradiated male rabbits (Fig. 3) or, in those instances where this " label " was not available, if restoration of the leucocyte and platelet counts to normal occurred within 7–12 days (Fig. 5). The percentage of animals showing such a rapid repopulation of their depleted bone

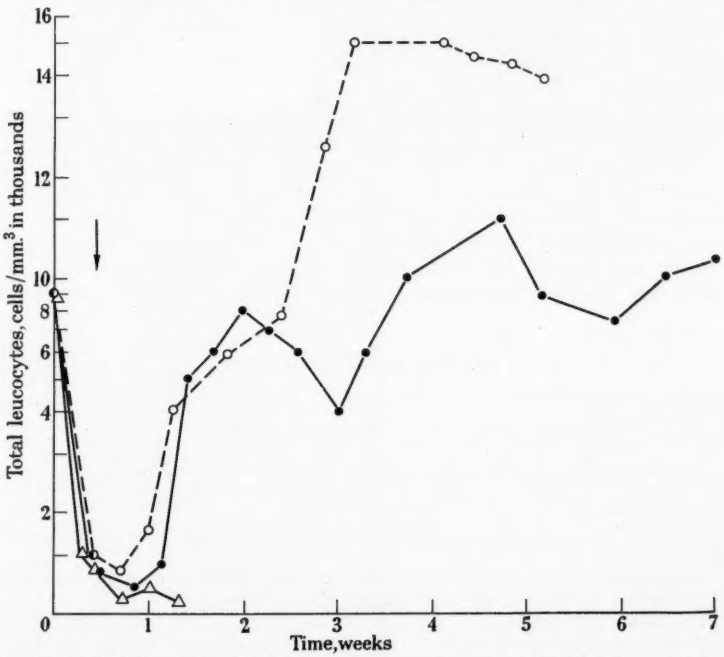


FIG. 5.—Effect of adult and foetal haemopoietic transplants on the total leucocyte counts of representative rabbits after 1600 r whole body X-irradiation.

△——△ Control animal.
○——○ Adult bone marrow treated animal which later developed fatal " secondary disease " with a typical terminal leucocytosis.
●——● Animal treated with 20-day foetal liver.

The arrow shows the day when the homologous cell suspension was given.

marrow spaces by donor type cells was related principally to the number of nucleated haemopoietic cells injected (Table I). Thus the more cells administered the higher the percentage of initially successful transplants. The exception was Group III, but here it is probable that the majority of the haemopoietic cells were relatively mature and that the number of cells capable of rapid reproduction was less than in Group V*a*.

Weight

Fig. 6 shows the weight curves for typical animals from some of the groups listed in Table I. The irradiated but otherwise untreated control rabbits steadily lost weight until all were dead. Animals in the groups treated with various haemopoietic tissues lost weight during the first 9–10 days post-irradiation, but this was followed by a recovery which was permanent for the animals receiving foetal liver. However, in many of the group given adult bone marrow and some of the group given neonatal liver, recovery was brief. At any time between the 16th and 40th day weight loss recurred marking the onset of " secondary disease ", and was followed by a steep fall in weight until death.

Causes of death

Table II gives a comparison of the causes of death in the various groups of irradiated rabbits.

Haemorrhage, particularly into the gut and occasionally into the lungs, was a major cause of death in the control animals. It occurred between the 6th and 11th days post-irradiation and coincided with the period of maximum depression of the platelet count. Most of the treated animals avoided or recovered from this complication because of the rapid output of platelets by the transplant.

Gastric perforation or rupture affected all the groups of rabbits given haemopoietic tissue and caused the death of between 15 and 23·3 per cent. Most of the controls died too soon from other causes for this complication to have appeared.

EXPLANATION OF PLATES

FIG. 1.—Femoral bone marrow from normal rabbit. Haematoxylin and eosin. ×480.

FIG. 2.—Liver from a normal 20-day rabbit foetus. Active widespread haemopoiesis involving all types of blood cells is apparent. Haematoxylin and eosin. ×480.

FIG. 3.—Peripheral blood smear from an irradiated male rabbit 17 weeks after treatment with 20-day female foetal liver. A heterophil showing a nuclear " drumstick " characteristic of the female can be seen. Leishman's stain. ×1600.

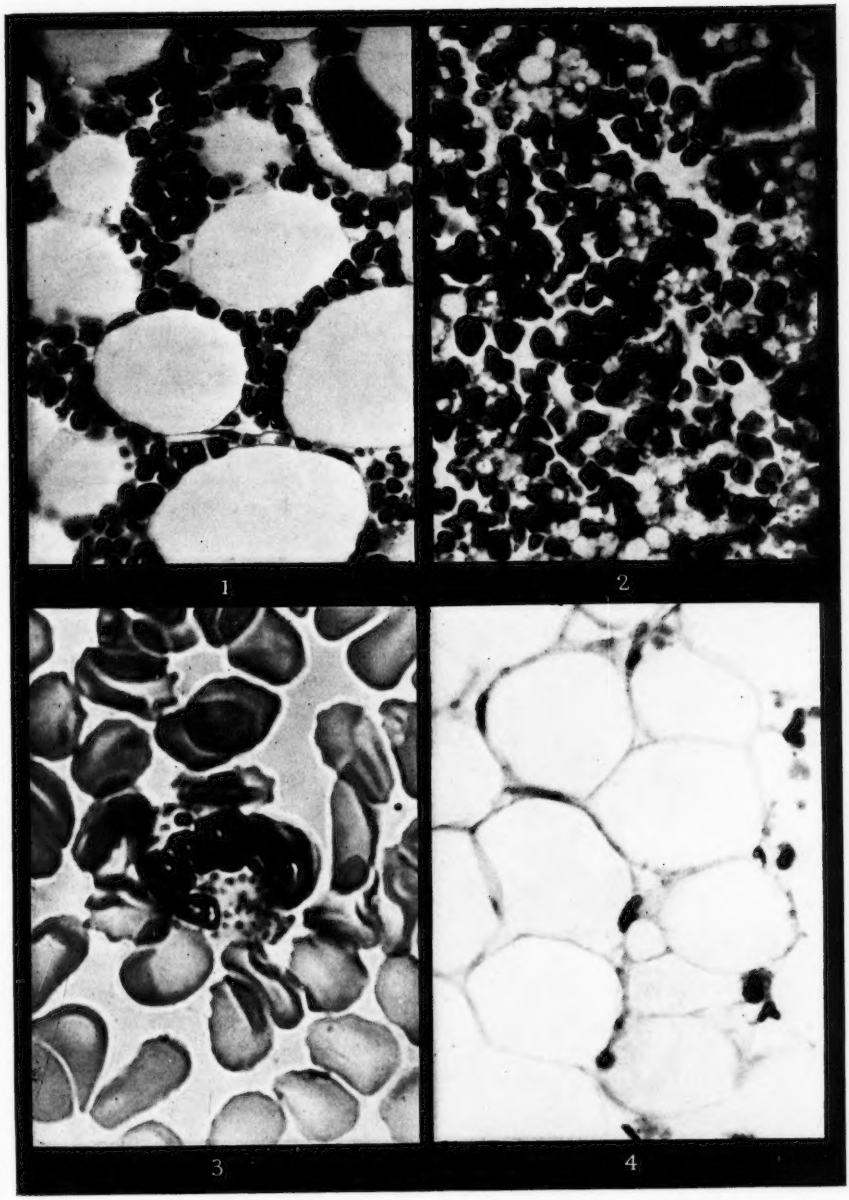
FIG. 4.—Aplastic bone marrow from a control animal that died 9 days after irradiation. Haematoxylin and eosin. ×480.

FIG. 7.—Successful marrow transplant in a 20-day foetal liver treated animal that died at 9 days following gastric rupture. Haematoxylin and eosin. ×480.

FIG. 8.—Femoral bone marrow from an adult marrow treated animal that died at 32 days after developing " secondary disease ". Marrow smears showed female cells. There is loss of fat. Haematoxylin and eosin. ×480.

FIG. 9.—Early repopulation of lymphoid nodule with lymphocytes and plasma cells in a 20-day foetal liver-treated animal that died at 9 days following gastric rupture. Haematoxylin and eosin. ×250.

FIG. 10.—Loss of lymphocytes in spleen of adult marrow treated rabbit with " secondary disease ". The lymphoid nodule is replaced by fibrous tissue and a few scattered plasma cells. Haematoxylin and eosin. ×250.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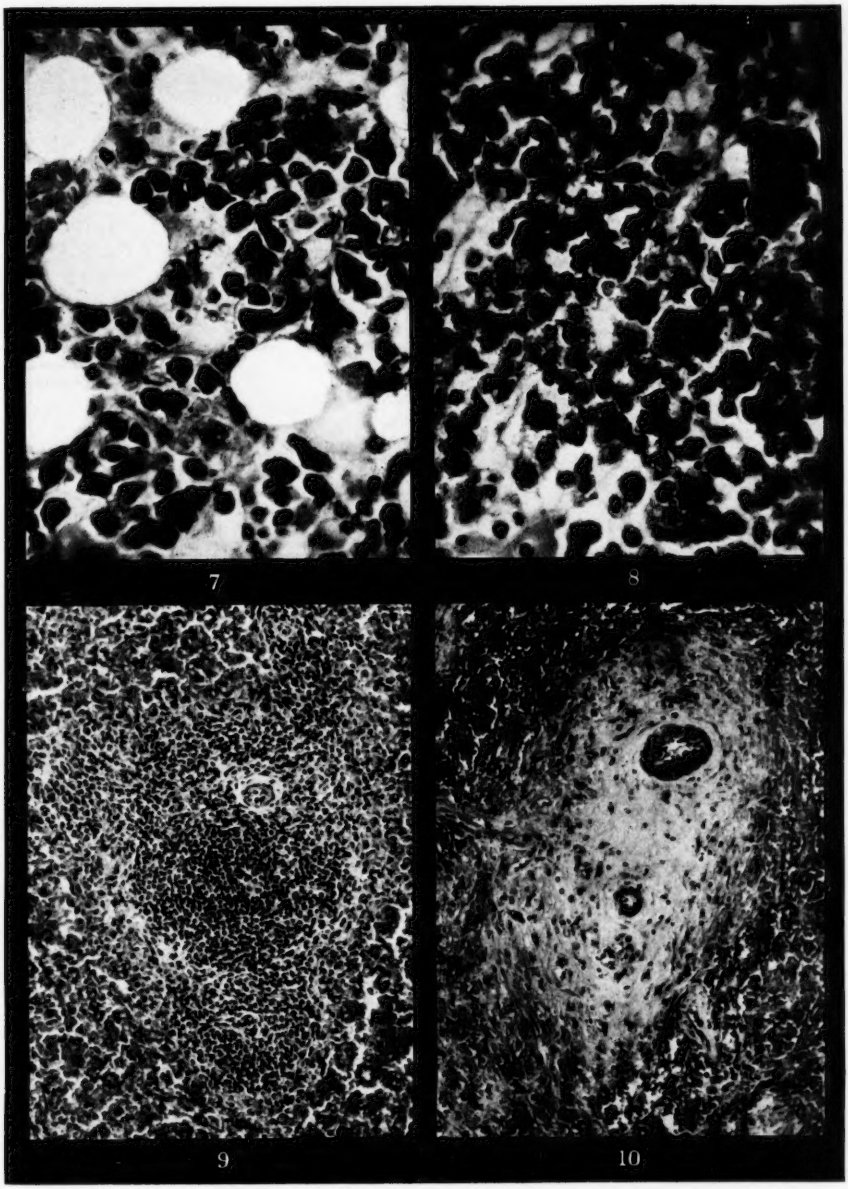

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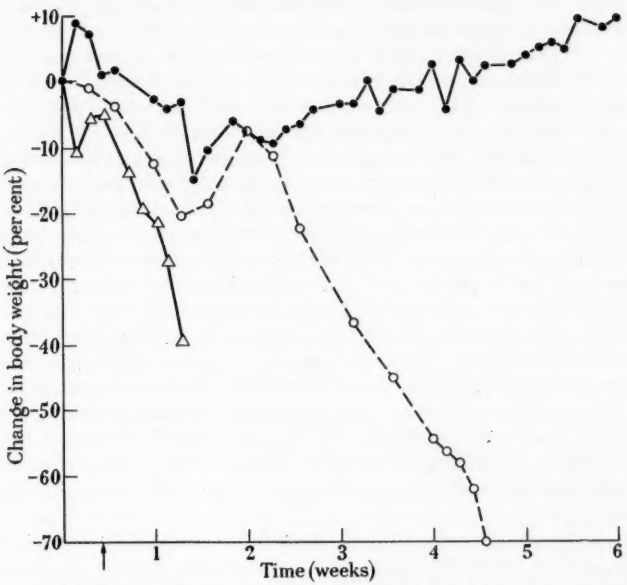

FIG. 6.—Weight curves of irradiated rabbits treated with adult and foetal haemopoietic tissue.

△———△ Control animal.
○———○ Adult bone marrow treated animal which later developed fatal " secondary disease ".
●———● Animal treated with 20-day foetal liver.

The arrow shows the day when the homologous cell suspension was given.

TABLE II.—*Comparison of Causes of Death in Irradiated Rabbits Treated with Various Homologous Haemopoietic Tissues*

Group	Haemopoietic tissue used in treatment	Percentage dying from: Haemorrhage	Gastric perforation or rupture	Broncho-pneumonia	Rejection of graft by host	Reaction of graft against host, " secondary disease "
I	None	65	0	35	—	—
II	Adult bone marrow	0	20	5	15	35
III	Newborn liver	20	15	5	10	20
IV	27-day foetal liver	6·7	23·3	3·3	16·7	0*
VA	Liver from one 20-day foetus	10	15	15	15	0
VB	Liver from two 20-day foetuses	15	15	10	5	0

* But 4 animals (13·3 per cent) went through a phase of wasting that resembled the onset of " secondary disease ". Recovery followed.

In the majority the lesion was a perforated peptic ulcer usually situated at the pylorus and almost always causing death at the 13th day. Less commonly the whole fundus of the stomach became thinned and ruptured generally between the 8th and 12th days. Peptic ulceration without perforation was fairly common.

Bronchopneumonia.—A massive necrotic and haemorrhagic non-purulent infection of the lungs by gram-negative bacilli killed many of the control animals, but a true patchy purulent peribronchiolar pneumonic consolidation also occurred in animals with successful marrow transplants. It was, of course, particularly common in animals in which an immunological battle was being fought, whether this was host rejecting graft or donor cells reacting against host antigens. However, these chimaeras were more than usually susceptible to respiratory infections and animals apparently doing well would occasionally suddenly develop pneumonia and die, usually in the first 3 weeks after irradiation.

Rejection of graft by host.—Of those animals with an initially successful haemopoietic transplant between 5 and 16·7 per cent later rejected the graft and died. In these cases after early restoration of the blood picture to normal, a progressive fall in the heterophils, platelets and packed cell volume occurred. Death came between the 21st and 27th days after irradiation, the final episode being haemorrhage or infection. At necropsy the bone marrow was aplastic, but the lymphoid tissue throughout the body was well regenerated and almost back to normal appearance.

Reaction of graft against host ("secondary disease").—Fatal "secondary disease" was found only amongst the rabbits treated with adult bone marrow or neonatal liver (Groups II and III). The percentage dying from this cause was higher when the foreign tissue was adult marrow than when it was new-born liver. Of the animals given 27-day foetal liver (Group IV) 13·3 per cent went through a phase of weight loss and diarrhoea that closely resembled the onset of "secondary disease", but subsequently, instead of progressing to emaciation and death, they made a slow recovery. The group given 20-day foetal liver avoided this complication completely. As noted previously (Porter and Murray, 1958*a*) the victims of "secondary disease" continued to show female cells in the blood smears indicating an intact and functioning marrow transplant. At necropsy the bone marrow was well repopulated (Fig. 8) and even hyperplastic, but there was widespread lymphoid atrophy (Fig. 10) and a patchy plasma cell infiltration of the frequently enlarged spleen. These features sharply differentiated this condition from that produced by host rejection of the graft.

DISCUSSION

The success of foetal as opposed to neonatal or adult haemopoietic tissue in preventing late deaths in lethally X-irradiated rabbits might theoretically be due to antigenic immaturity of the donated cells. At present little is known about the development of antigenic maturity in higher animals but it seems from grafting experiments (Cannon, Terasaki and Longmire, 1957) that there is reduced antigenicity of new-born as compared with 14-day chick skin. This hypothesis, however, seems untenable in the present experiments as in all the treated groups a fairly constant percentage of animals died as the result of unequivocal rejection of the transplant by the host.

More probably success is due to the immunologically immature transplanted

foetal cells becoming, as they mature, tolerant of their homologous host's antigens. Billingham, Brent and Medawar (1953) have described how injection of foetal mice with living adult homologous cells will induce a high degree of tolerance of subsequent skin grafts from the donor strain. This occurs because the immature immune system of the foetus is unable to reject the foreign cells which persist giving rise to a chimaera (Billingham and Brent, 1957).

In the present experiments foetal reticuloendothelial and lymphoid cells are exposed to adult antigens within the irradiated adult host, whereas in the experiments of Billingham and Brent the foetus is exposed to adult antigens in utero. In both these instances a chimaera is produced and it seems probable that tolerance to the adult cells also results in both cases.

There is considerable evidence that developing animals are poor producers of antibody, but immense differences exist between species. In the rabbit induction of antibody formation to protein antigens is very difficult in the new-born (Smith and Bridges, 1956); but classical immunological maturity does not necessarily coincide with transplantation immunological maturity. For instance, Egdahl (1957) has found that rabbit foetuses grafted with homologous skin 1–3 days before delivery reject such grafts at about the same rate as do adult animals. Likewise the occurrence in the present experiment of fatal " secondary disease " in animals treated with new-born liver, and transient evidence of the same syndrome in a few of those treated with 27-day foetal liver, indicates that at some time between the 20th day and 27th day of pregnancy an important degree of transplantation immunological maturity occurs in the rabbit. That maturation is still incomplete at birth is shown by the lower incidence of " secondary disease " after neonatal liver than after adult bone marrow treatment.

In these experiments an LD_{100} 30 days dose of radiation was used in a vain effort to eliminate the ability of some of the irradiated animals to regenerate their own immune systems and reject the transplants. It is interesting that Barnes, Ford, Gray and Loutit (1958), using another cytological method, have also noted in some of their homologous chimaeras a reversion of the marrow cells back to host type. As they suggest, it is apparently the cell clones with the greatest physiological competence, whether they be of donor or host origin, which ultimately predominate. It should perhaps be noted that all transplants in the present experiments were into male hosts. Thus any possible complication from the known failure of some grafts from a male donor to a female host (Eichwald and Silmser, 1956) was avoided.

Recently a few reports (Thomas, Lochte, Lu and Ferrebee, 1957; Humble and Newton, 1958) have appeared of attempts to treat marrow aplasia in humans by haemopoietic, usually adult bone marrow, transplantation. If in such experiments the host's immune system is incompletely paralysed rejection of the marrow graft is to be expected; if on the other hand suppression of the host's defences is adequate and a successful transplant is achieved then the hazard of a late graft-versus-host reaction (" secondary disease ") is inevitably present. It would now seem that in any future therapeutic attempts to create human chimaeras the use of early foetal haemopoietic tissue, by preventing " secondary disease ", offers most hope of lasting recovery. Difficulties of supply of such tissue might be partly overcome by storage of cell suspensions at $-70°$ by the Polge-Parkes glycerol freezing method (Barnes and Loutit, 1955; Porter and Murray, 1958*b*).

SUMMARY

Late deaths in lethally irradiated rabbits treated with homologous haemopoietic tissue can be avoided if 20-day foetal liver instead of adult bone marrow is used to produce the chimaeras.

Twenty-seven day foetal liver is not quite so effective, and new-born liver is little better than adult tissue, in preventing late mortality.

Success is probably due to the immunologically inactive transplanted foetal lymphoid and reticuloendothelial cells becoming, as they mature, tolerant of their homologous host's antigens.

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THE EFFECT OF *MYCOBACTERIUM TUBERCULOSIS* (BCG) INFECTION ON THE RESISTANCE OF MICE TO BACTERIAL ENDOTOXIN AND *SALMONELLA ENTERITIDIS* INFECTION

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ACTIVATION of the reticulo-endothelial system (RES) has been associated in recent years with 2 interesting phenomena—the development of tolerance to bacterial endotoxins and the stimulation of non-specific immunity to infection. For a week or so after the injection of endotoxin, animals are more resistant to the lethal or other effects of a second dose (Thomas, 1954). A time relationship has been observed between the development of tolerance and an increase in phagocytic function of the RES (Biozzi, Benacerraf and Halpern, 1955). The importance of the RES in the body's defences against endotoxin has been emphasised in a review by Thomas (1957). Secondly, a generalised increase in resistance to many bacterial infections, which follows the injection of certain colloidal materials, has been considered also to be correlated with activation of the RES produced by injected colloid (Biozzi, Halpern, Benacerraf and Stiffel, 1957; Howard, Rowley and Wardlaw, 1958). The duration of most of these examples of non-specific immunity has been a few days only.

The infection of mice with *Mycobacterium tuberculosis* (strain BCG) leads to a more intense and long-lasting stimulation of the phagocytic activity of the RES than can be accomplished by a single injection of colloid (Biozzi, Benacerraf, Grumbach, Halpern, Levaditi and Rist, 1954). The effect is maximal 2–3 weeks after intravenous injection of the bacteria. This infection does not produce any mortality in mice. The relationship between the phagocytic function of the RES and resistance to endotoxins is more complex than appeared at first. It has been shown recently that mice infected 2 weeks previously with BCG are extremely susceptible to the lethal action of endotoxin contained in *Salmonella typhi* (Halpern, Biozzi, Howard, Stiffel and Mouton, 1958). Similar findings have been described by Suter, Ullman and Hoffman (1958).

This communication describes changes in the susceptibility of mice to endotoxin and to infection by *Salmonella enteritidis* in BCG-treated mice, together with changes in the phagocytosis of isotopically-labelled endotoxin and bacteria by the RES of such animals. The effect of BCG on one aspect of intracellular metabolic activity, the catabolism of ^{131}I-labelled heat-denatured albumin by Kupffer cells, has also been investigated.

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MATERIALS AND METHODS

Mice.—Male animals of a Swiss albino strain, weighing 22–28 g. were used throughout.

BCG infection.—The Pasteur Institute strain of *Myco. tuberculosis* (strain BCG) was used. Eight day cultures in Dubos medium were kindly provided by Dr. F. Grumbach. Mice were given a dose of 0·25 mg. wet weight intravenously into a tail vein. The various investigations were made on the animals 12–14 days later.

Determination of the phagocytic activity of the RES.—The kinetics of blood clearance of a standard dose of colloidal carbon (16 mg./100 g., preparation C11/1431a) was measured as described by Biozzi, Benacerraf, Stiffel and Halpern (1954). The " phagocytic index " K is an expression of the whole activity of the RES and the " corrected phagocytic index " α is a measure of this function per unit weight of hepato-splenic tissues.

Endotoxins.—Two preparations were used : a suspension of *Salm. typhi* killed by heating at 60° for 1 hr. and the purified lipopolysaccharide from *Bacterium coli* (strain 2380). We are indebted to Dr. Derrick Rowley for a supply of this latter material. Both preparations were suspended in saline and volumes of 0·2 ml. per 20 g. injected intravenously into the dorsal vein of the penis.

^{32}P-labelled lipopolysaccharide (^{32}P–*LP*) was prepared from *Bact. coli* (strain 2380) grown on ^{32}P containing medium by the method of Rowley, Ali and Jenkin (1958). We are indebted to Mr. W. Ali for a batch of this material, supplied as a suspension containing 4 mg. per ml. Its initial activity was 2310 counts per min. per µg. Fresh dilutions, made in saline before use, were always injected intravenously. To measure the rate of clearance of ^{32}P–LP, frequently repeated samples of 0·025 ml. blood were removed from the retro-orbital venous plexus and dried on filter paper discs. The activity of these samples was read under an end-window Geiger counter. The mice were killed 30 min. after injection by decapitation, the liver, spleen, lungs and kidneys were removed and homogenized in saline. The radioactivity of aliquots of these was counted as for the blood samples.

Salmonella enteritidis (var. Danysz) was obtained from Dr. Le Minor. Overnight broth cultures were subcultured into nutrient broth, incubated for 6 hr. at 37° and diluted in saline. The concentration of organisms was calculated from the optical density in a photo-electric absorptiometer. Mice were infected with an intravenous dose of 1000 bacteria. The number of deaths was recorded daily.

^{51}Cr-labelled suspensions of Salm. enteritidis.—Cultures which had been grown for 18 hr. on nutrient agar at 37° were washed off with the minimum quantity of saline, filtered through glass wool and washed twice. Volumes of 40–50 ml., containing about 4×10^{10} bacteria per ml., were sterilised by heating at 60° for 90 min. To 19 ml. of *Salm. enteritidis* suspension 3 mc. of ^{51}Cr (3 ml. of a solution of sodium chromate containing 29 µg. Cr per ml. pH 8·0) was added and the mixture incubated for 48 hr. at 37°. The bacteria, when washed 3 times with saline, were found to have fixed 60 per cent of the ^{51}Cr. Less than 2 per cent free ^{51}Cr was present in these washed suspensions, which were stored at 4°.

The clearance from the circulation of ^{51}Cr-*Salm. enteritidis*, after intravenous injection, was followed by removing 0·025 or 0·05 ml. successive blood samples from the retro-orbital venous plexus and lysing them in 3 ml. of 0·1 per cent sodium carbonate. The activity of these samples was read using a well type scintillation counter. Whole organs, or weighed samples of them, were placed in small plastic dishes and their radioactivity measured with the same scintillation counter, keeping the geometry constant.

Determination of the metabolic activity of Kupffer cells on ^{131}I-labelled heat-denatured albumin (CA^{131}I).—CA^{131}I containing 7 per cent of Iodine was prepared by the method described by Benacerraf, Biozzi, Halpern, Stiffel and Mouton (1957). The rate of catabolism of CA^{131}I within Kupffer cells was calculated as previously described by measuring the decreasing curve of hepatic radioactivity after fixation of a given amount of CA^{131}I in the Kupffer cells. The initial rate of this reaction was calculated from the linear function between hepatic radioactivity and time which characterises the first part of the curve (Biozzi, Halpern, Stiffel and Mouton, 1958).

RESULTS

The stimulating effect of BCG on the phagocytic activity of the RES was confirmed. Table I shows a comparison between the clearance of colloidal

carbon (16 mg. per 100 g.) in controls and mice infected with BCG 14 days previously. The phagocytic index "K" was increased five-fold and the ratio $\frac{W}{WLS}$ halved in the treated group. In view of the great increase in size of the liver and spleen in these mice, the corrected phagocytic index "α" did not change.

TABLE I.—*The Effect of BCG Infection on the Phagocytic Activity of the RES*

	Number of animals	Phagocytic index K	$\frac{W^*}{WLS}$	Corrected phagocytic index α
Mice infected with BCG 14 days previously	11	0·072	9·3	3·9
Controls	7	0·015	17·2	4·2

*W = body weight.
WLS = weight of liver and spleen.

The effect of BCG infection on susceptibility to endotoxin

Mice infected with BCG 13 days previously were found to be far more sensitive to killing by endotoxin than normal. In one group of experiments a suspension of killed *Salm. typhi* was used as the toxin (Table II). The calculated LD_{50}'s were $4{\cdot}5 \times 10^7$ bacteria for the BCG-treated mice and 5×10^9 for the controls—a hundred-fold increase in susceptibility. It is noteworthy that the dose response was much steeper in the controls than in the treated group. Similar results were obtained when the toxin used was the purified lipopolysaccharide from *Bact. coli* rather than a crude bacterial suspension (Table III). In this experiment, the LD_{50} of the toxin was 250 μg. for normal mice and 3·2 μg. for BCG-infected mice. Again the dose response was steeper in the control group.

TABLE II.—*A Comparison of the Toxicity of a Suspension of Killed* Salm. typhi *for BCG-infected and Normal Mice*

	Dose of killed *Salm. typhi* per 20 g. (intravenously)	Number of deaths / Number of mice injected	Percentage mortality
Mice infected with BCG 13 days previously	1×10^6	0/5	0
	5×10^6	10/29	34
	2×10^7	11/26	42
	5×10^7	4/9	44
	2×10^8	14/20	70
	5×10^8	4/5	80
	2×10^9	5/5	100
Controls	2×10^9	0/13	0
	5×10^9	10/16	62
	1×10^{10}	22/22	100

The rate of clearance of ^{32}P-labelled lipopolysaccharide was investigated, in case the stimulation of the RES previously shown with carbon had no effect on this material. The results in Fig. 1 show that ^{32}P–LP was phagocytosed more rapidly in the BCG-infected mice. A unique feature of lipopolysaccharide in studies of this kind is that its clearance does not follow an exponential function

TABLE III.—*A Comparison of the Toxicity of* Bact. coli *Lipopolysaccharide for BCG-infected and Normal Mice*

	Dose of *Bact. coli* lipopolysaccharide per 20 g. (μg., intravenously)	Number of deaths / Number of mice injected	Percentage mortality
Mice infected with BCG 14 days previously	0·1	0/6	0
	0·5	1/6	17
	2·0	3/9	33
	5·0	4/6	67
	10·0	5/6	83
	20·0	6/6	100
Controls	100	1/8	13
	200	4/13	31
	400	6/8	75

like other colloids, but is continually slowing almost from the moment of injection both in control and treated animals. It is thus impossible to express these results as phagocytic indices in the usual manner. However, 10 min. after

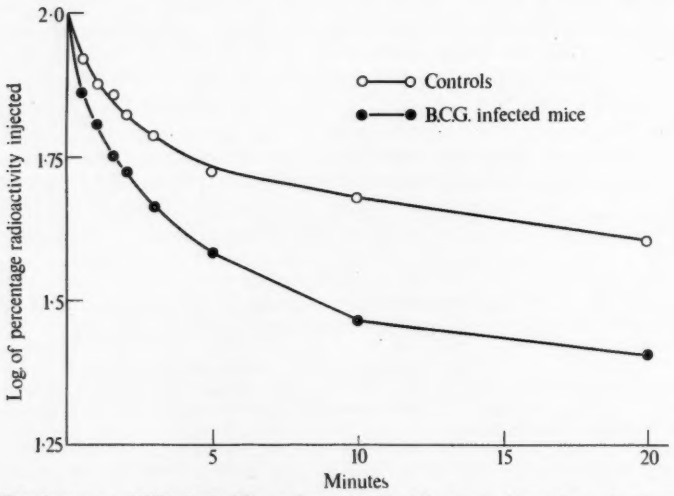

FIG. 1.—The clearance of ^{32}P-labelled lipopolysaccharide (40 μg. per 20 g.) from the circulation in normal mice and mice infected with BCG 14 days previously. Each curve represents the mean of 7 animals.

injection, 50 per cent of the ^{32}P–LP had been removed from the circulation of control mice as compared with 71 per cent in BCG-treated mice. The mean distribution of radioactivity 30 min. after injection of ^{32}P–LP is shown in Table IV. Slightly greater amounts were present in the liver and spleen of the treated mice. Thus, in spite of the fact that intravenously injected endotoxin is cleared more efficiently in BCG-treated mice, they are a hundred times more sensitive than normal to its lethal effect.

TABLE IV.—*Distribution of* ^{32}P*-lipopolysaccharide, 30 min. after an Intravenous Dose of 40 μg. per 20 g.*

	Percentage					
	Liver	Spleen	Lungs	Kidneys	Blood	Total
Mice infected with BCG 14 days previously	48	6	2·8	2	24	82·8
Controls	42·2	2·4	1·9	2·1	35	83·6

The effect of BCG infection on resistance to Salm. enteritidis *infection*

Notwithstanding their greatly increased sensitivity to endotoxin, BCG-treated mice were found to be more resistant to infection with relatively small doses of an endotoxin-forming organism—*Salm. enteritidis*. The results of a typical experiment are represented graphically in Fig. 2. No ultimate survivors

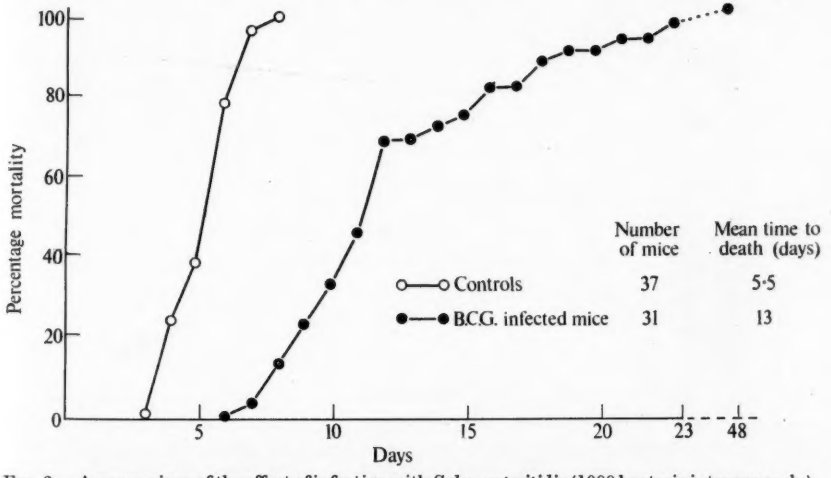

FIG. 2.—A comparison of the effect of infection with *Salm. enteritidis* (1000 bacteria intravenously) in normal mice and mice infected with BCG 13 days previously.

were obtained in either control or treated mice, but the mean survival time was greatly increased in the latter group. Clearly, resistance to endotoxins and the prevention of bacterial multiplication rising to a lethal level involve quite separate mechanisms. A close correlation was found between changes in the phagocytic index " K " and the resistance to *Salm. enteritidis* infection, after varying periods of BCG infection (Fig. 3). On the other hand, this figure shows that the resistance to endotoxin was inversely related to these values throughout.

In parallel with this enhanced resistance to *Salm. enteritidis*, it was found that the initial rate of removal from the circulation of ^{51}Cr-labelled suspensions of this strain was twice as rapid as normal (Fig. 4). The bacteria were cleared according to an exponential function for about the first 5 min., when the rate started to slow progressively. The phagocytic indices (0·050 for controls and 0·106 for BCG-treated mice) were calculated from the exponential period. The

mean distribution of radioactivity 30 min. after injection of the tagged bacteria is shown in Table V.

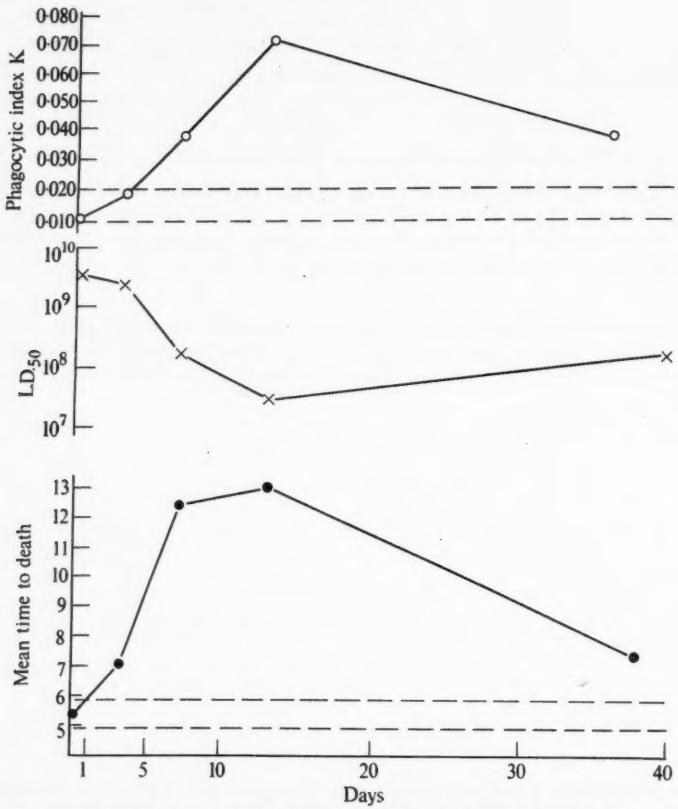

FIG. 3.—Changes in (*a*) the phagocytic index (K) of the RES, (*b*) resistance to endotoxin (LD_{50} of killed *Salm. typhi* per 20 g.), and (*c*) resistance to infection with *Salm. enteritidis* (mean survival time following 1000 organisms intravenously), at different times after infection with BCG in mice.

The broken lines indicate the standard deviations of control groups tested at the same time as treated animals. The phagocytic indices represent the means of groups of 6–8 mice. The LD_{50}'s were calculated from groups of 12 mice injected with different dosages. The mean survival times were established with groups of 15 mice.

TABLE V.—*Distribution of* ^{51}Cr-Salm. enteritidis, 30 *min. after an Intravenous Dose of* 4×10^9 *Bacteria per* 20 *g.*

	Percentage					
	Liver	Spleen	Lungs	Kidneys	Blood	Total
Mice infected with BCG 14 days previously	87	7·4	1·7	0·4	6	102
Controls	74·5	4·5	4·1	0·3	10	93·4

The effect of BCG-infection on the rate of breakdown of $CA^{131}I$ by Kupffer cells

The data presented in Table VI indicate that the maximum quantity of $CA^{131}I$ phagocytosed by the Kupffer cells was about the same in both BCG-infected and control mice, at a time when the injected colloid had been almost completely cleared from the blood. On the other hand, the rate of breakdown of $CA^{131}I$ was markedly increased in mice infected with BCG. It may be calcu-

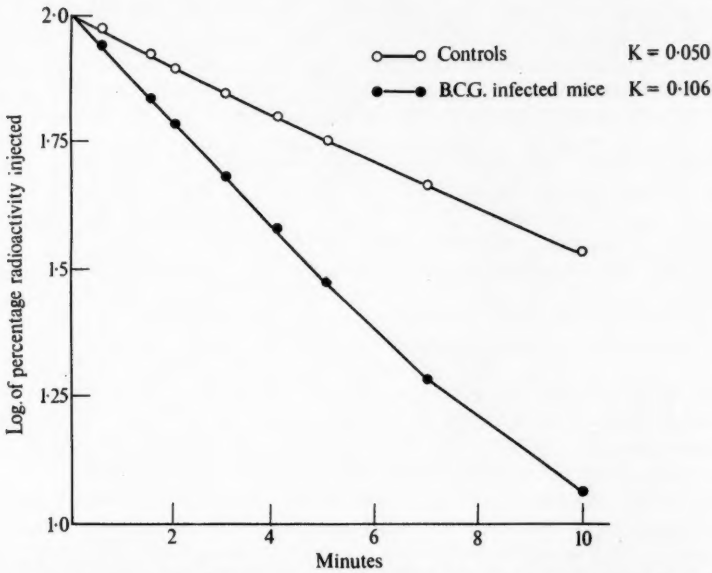

FIG. 4.—The clearance of ^{51}Cr-labelled *Salm. enteritidis* ($4{\cdot}0 \times 10^9$ bacteria per 20 g.) from the circulation in normal mice and mice infected with BCG 14 days previously. The curves represent the means of 11 and 10 animals respectively.

lated from data previously reported (Biozzi *et al.*, 1958) that the amount of $CA^{131}I$ localised in the Kupffer cells of control mice would need to be increased to 630 μg. to obtain a rate of breakdown similar to that observed in the BCG-infected mice with a dose of 387 μg.

TABLE VI.—*A Comparison of the Initial Rate of Breakdown of $CA^{131}I$ by Kupffer Cells in the Livers of Normal and BCG-infected Mice*

	Dose of $CA^{131}I$ injected intravenously	Maximum quantity of $CA^{131}I$ fixed in the liver (μg.)	Initial rate of breakdown of $CA^{131}I$ (μg. per min.)
Mice infected with BCG 13 days previously	500	387	6·8
Controls		372	4·6

DISCUSSION

At first sight, our finding that BCG-infected mice show a greatly increased susceptibility to endotoxin, in spite of stimulation of the RES, conflicts with current opinion on the basis of tolerance to endotoxins. There is, moreover, much evidence to support the view that the degree of resistance to endotoxins can be correlated with the functional activity of the RES. Firstly, ^{32}P-labelled endotoxin is phagocytosed rapidly by the RES after intravenous injection in mice (Howard *et al.*, 1958). Secondly, for many hours after the injection of a " blockading " dose of Thorotrast, trypan blue, colloidal carbon or saccharated iron oxide, the susceptibility of animals to the lethal effect of endotoxin is greatly enhanced (Bennett, 1952 ; Good and Thomas, 1952 ; Smith, Thomas and Good, 1953). Thirdly, within about 24 hr. of a non-lethal injection of one endotoxin, animals become resistant to several lethal doses of any other endotoxin (Zahl and Hutner, 1944 ; Thomas, 1954). Such " tolerance " has been associated with an increase in the phagocytic activity of the RES (Biozzi *et al.*, 1955 ; Benacerraf and Sebestyen, 1957). Finally, tolerance may be abolished by subsequently blockading the RES (Beeson, 1947*a*, *b*).

There are, nevertheless, certain objections to a straightforward association between resistance to endotoxins and the extent of phagocytic function of the RES. The degree of endotoxin tolerance usually obtained will protect against 3–4 lethal doses, and the increase in phagocytic activity of the RES is of the same order of magnitude. On the other hand, " blockaded " animals may be a thousand times more sensitive to endotoxin than normal (Good and Thomas, 1952), whilst phagocytic activity rarely falls below 25 per cent. Again, attempts to produce tolerance to endotoxin by activating the RES with saccharated iron oxide or Thorotrast have failed (Biozzi and Howard, unpublished).

These facts, together with our new finding that BCG-infected mice are a hundred times more sensitive to endotoxin than normal permits either of two conclusions. Resistance to endotoxin may not be associated with the functional activity of the RES ; or alternatively, a causal relationship may exist, but in certain circumstances such as BCG infection, a greatly increased sensitivity at the site of action of endotoxin may obliterate any potential protective effect of the RES. Thus, as we have shown in BCG-treated mice, the rate of clearance of endotoxin is enhanced, but the smaller quantity circulating might still be adequate to initiate fatal intoxication in a hypersensitive animal.

Several examples have now been recorded of the inhibitory effect of tuberculous infection on the development of a secondary, heterologous infection. Pullinger (1936) found that guinea-pigs were more resistant to brucellosis if they were infected simultaneously with *Myco. tuberculosis*. Tuberculous guinea-pigs have been found also to be relatively resistant to *Bacillus anthracis* and *Brucella suis*, provided that both the primary and secondary pathogen were introduced via the same pulmonary route (Henderson, Lancaster, Packman and Peacock, 1956). Mice infected with *Myco. tuberculosis* (strain BCG) became more resistant than normal to *Staph. aureus* (Dubos and Schaedler, 1957), whilst mice were found to be highly resistant to *Pasteurella pestis* when fatally infected with *Myco. tuberculosis* (strain H_{37} Rv) (Girard and Grumbach, 1958). In our experiments, the survival time following *Salm. enteritidis* infection was clearly increased in BCG-carrying mice, although there was no improvement in the prospect of

ultimate survival. This increase in resistance is, nevertheless, as good as that against *Salm. typhimurium* (a similar infection) following either specific active immunisation (Hobson, 1957) or stimulation of non-specific immunity by Lipid A (Howard, Rowley and Wardlaw, 1957).

Our experiments with isotopically-labelled materials have shown that the rates of clearance of both bacteria and endotoxin, like that of carbon, were increased in BCG-infected animals. A phenomenon emerged, however, which has not been experienced previously in studies on the phagocytosis of colloids by the RES. The rate of clearance of ^{51}Cr-labelled *Salm. enteritidis* was constant in semi-logarithmic plot for the first few minutes and then started to become progressively slower in both normal and treated animals. With ^{32}P–LP, clearance was never exponential, but showed continual retardation right from the time of injection. The significance of this effect will be considered in a later publication.

Finally, one may question whether an increased rate of phagocytosis of bacteria by the RES is sufficient by itself to explain a rise in resistance to bacterial infection. It is surely more likely that when the bacteria can survive within RE cells, as in tuberculous and typhoid-like Salmonella infections, their intracellular multiplication must be either prevented or retarded for any enhancement of resistance to appear. The mechanism of bacterial killing by macrophages is unknown. In BCG-infected mice the production of specific antibody following the injection of killed Gram-negative bacteria is greater and longer lasting than in controls (Halpern, Biozzi, Stiffel and Mouton, 1958). On the other hand, our results on the breakdown of CA^{131}I show quite clearly that apart from stimulating phagocytic activity, BCG infection enhances certain metabolic activities of RE cells. Possibly, changes of this nature may reflect the development of an intracellular environment unfavourable for bacterial multiplication.

SUMMARY

Two weeks after infection with *Myco. tuberculosis* (strain BCG) the rate of clearance of carbon from the blood stream by the RES in mice was increased 5 times. Simultaneously, the animals were 100 times more susceptible to killing by bacterial endotoxin than normal. Endotoxin labelled with ^{32}P was, like carbon, cleared more rapidly from the circulation. Its rate of removal was at no time exponential, however, but showed continual retardation in both treated and control mice.

Although BCG-infected mice were hypersusceptible to endotoxin, they were more resistant than normal to infection with *Salm. enteritidis*, as measured by the mean survival time. Suspensions of this organism labelled with ^{51}Cr were cleared from the blood twice as rapidly as normal in the BCG-treated animals. Their removal was exponential for the first 5 min. and then started to slow up.

The rate of catabolism of CA^{131}I by Kupffer cells was increased in the BCG-treated group.

The possible relationship of activation of the RES to the phenomena of tolerance to endotoxins and stimulation of non-specific immunity is discussed.

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THE INTERFERENCE OF MOUSE HEPATITIS VIRUS WITH ECTROMELIA IN MICE AND A POSSIBLE EXPLANATION OF ITS MECHANISM

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TREATMENT of mice with non-lethal doses of bacterial endotoxins and other agents which stimulate the activity of the reticulo-endothelial system lowers their resistance to the pathogenic effect of small and moderate doses of ectromelia virus (Gledhill, 1959*b*). Nevertheless, the inoculation of mice with serum from mice bled two hours after treatment with similar doses of bacterial endotoxin raises their resistance to the pathogenic effect of small doses of ectromelia (Gledhill, 1959*a*). In view of a suspected similarity between the effects on mice of bacterial endotoxin and mouse hepatitis virus (MHV1 : Gledhill, Dick and Niven, 1955 ; Dick, Niven and Gledhill, 1956), an agent which produces benign hepatitis in weanling mice, it seemed of interest to determine whether mice inoculated with this virus would resemble mice inoculated with endotoxin and exhibit less resistance to small doses of ectromelia than normal mice. Experiments are described which show that, on the contrary, prior infection with mouse hepatitis virus considerably reduces the mortality of mice infected with ectromelia and the reduction of mortality occurs even in mice infected with large doses of ectromelia. Since this elevated resistance towards ectromelia was found to be transient and no evidence of antigenic relationship between the viruses was observed, the sparing effect of MHV1 infection on ectromelia is considered to exemplify the phenomenon of interference. In an attempt to find a basis for the interference, we considered the possibility that infection with mouse hepatitis virus might reduce the pathogenicity of ectromelia by production of a substance similar to that present in the serum of mice 2 hr. after treatment with bacterial endotoxin. Evidence has been found which points to the existence of such a substance in the serum of mice infected with mouse hepatitis virus.

METHODS

Weanling mice aged 3 weeks (10–12 g.) were used in all experiments unless stated otherwise. Mice of the TO strain, bred at the National Institute for Medical Research, Mill Hill, since 1953, were used for the interference experiments. In the experiments to demonstrate the sparing effect of sera from mice infected with MHV1, mice of the VS strain were used since they are somewhat more susceptible to the lethal effect of ectromelia than TO mice (Gledhill, 1959*b*). For the preparation of the sera VS mice aged 10–12 weeks were used ; these are designated " adult VS mice " and for testing the sera weanling VS mice were used. Mice of the Princeton strain have been bred at the Harvard Hospital, Salisbury, since 1956. when a few were sent to us by Dr. J. B. Nelson of the Rockefeller Institute, New York. Weanling mice of this strain were used for the preparation of immune serum against MHV1 because it was desirable to use serum from mice devoid of antibody against MHV1 and the Princeton mice, unlike the VS and TO, are believed not to be carriers of MHV1.

Dilutions of viruses were made in 50 per cent serum broth-saline. MHV1 made in bulk as a 10 per cent liver-spleen suspension was titrated and stored in ampoules at −76° and was injected in a dose of 0·2 ml. as 1 per cent suspension; this represented about 10^3 ID_{50} for weanling VS mice. The strain of ectromelia had been isolated from a spontaneous outbreak in 1951 and after a few mouse passages was stored at −76°. This material served as seed for the preparation of the batches of virus used in the present experiments, each of which consisted of a 10 per cent liver-spleen suspension, titrated and distributed in 2 ml. amounts in screw capped bottles at −76°.

Viruses of the MHV group are poor antigens and immune sera against MHV1 have never shown satisfactory titres. The method of preparation of immune sera adopted had given some success in experiments with MHV1 and related viruses and was as follows. About 100 weanling female Princeton mice received 3 intraperitoneal (i.p.) injections of 10 per cent liver-spleen extract containing MHV1 at weekly intervals (0·2 ml., 0·5 ml. and 0·5 ml.) and 2 more at fortnightly intervals (0·5 ml.). During the 2 weeks in which no i.p. inoculation was given, the mice received intramuscularly into the thigh muscles of both legs 0·1 ml. of the MHV1 suspension added to 0·1 ml. of adjuvant mixture consisting of 9 parts Bayol F (Shell) and 1 part sorbitol mono-oleate (Watford Chemical Co. Ltd.). The mice were bled out 7 days after the last intraperitoneal injection. High titred serum against ectromelia was easily prepared by a single intraperitoneal inoculation of a liver suspension containing about 10^6 LD_{50} of ectromelia into TO mice recently recovered from the disease and bleeding them out 10 days afterwards. The sera were inactivated by heating at 56° for 30 min. and used in neutralization tests with both ectromelia and the lethal *E. coccoides*-MHV1 mixture (Gledhill, Dick and Andrewes, 1952; Niven, Dick, Gledhill and Andrewes, 1952). Decimal dilutions of each agent were made and volumes of each dilution were mixed with equal volumes of the homologous serum, the heterologous serum, normal mouse serum and serum broth-saline. Each mixture was inoculated intraperitoneally into groups of 5 mice in a dose of 0·2 ml. after standing at laboratory temperature (20°–25°) for 30–40 min. The LD_{50} was calculated by the method of Reed and Muench (1938) from the specific mortality in each of the 8 titrations.

RESULTS

The Interference of Mouse Hepatitis Virus (MHV1) with Ectromelia

A 1 per cent mouse liver suspension containing about 10^3 LD_{50} MHV1 was injected into a group of 20 weanling TO mice and another group of 20 similar mice was kept as a control. After 24 hr., both groups were injected intraperitoneally with a liver suspension containing 30 LD_{50} of ectromelia. It will be seen from Table I that a significantly higher proportion of the mice pretreated with mouse hepatitis virus survived and the average survival time of those which died was significantly longer. In another experiment, ectromelia was inoculated intraperitoneally in decimal dilutions from 10^{-1} to 10^{-9} into groups of 6 normal mice and into similar groups of mice which had been inoculated intraperitoneally 24 hr. previously with the above dose of MHV1. For the pretreated mice the total mortality was 13/54 and the value of $-\log LD_{50}$ was 2·5 as against a total mortality of 40/54 and $-\log LD_{50}$ of 7·3 for the normal mice. Thus,

$$\log \frac{LD_{50}\ \text{pretreated mice}}{LD_{50}\ \text{normal mice}}$$

had a value of 4·8 and this difference represents a marked reduction in the pathogenicity of ectromelia over a wide range of doses.

Although the properties of mouse hepatitis virus and ectromelia would not suggest the likelihood of antigenic relationship between them, an experiment was undertaken to eliminate this possibility. The results of the experiment (carried out as described above) are shown in Table II. It will be seen that the

TABLE I.—*Sparing Effect of Mouse Hepatitis Virus (MHV1) Inoculated (i.p.) 24 hr. before Inoculation of Ectromelia (30 LD_{50} i.p.)*

Pre-inoculated with MHV1 (+ −)	Survivors Mice in group	Average survival time of mice which died (days)
+	14/20	15·2±2·6
−	3/20	11·3±2·2

10^3 LD_{50} MHV1 inoculated (0·2 ml. i.p.) 24 hr. before 30 LD_{50} ectromelia (0·2 ml. i.p.).
Survivor ratio 14/20 is significantly greater than 3/20 ($\chi^2 = 10·2$, $n = 1$, $p \doteq 0·001$).
Mean survival time 15·2 is significantly greater than 11·3 ($t = 3·57$, $n = 21$, $p < 0·001$).

TABLE II.—*Neutralization of MHV1 and Ectromelia by their Antisera and Normal Mouse Serum*

Neutralization with	Infected with *E. coccoides*-MHV1 (− log LD_{50})	Neutralization index	Mortality ratio in titration	Infected with Ectromelia (− log LD_{50})	Neutralization index	Mortality ratio in titration
MVH1 antiserum	5·6	**1·8**	16/35	8·8	0·6	32/35
Ectromelia antiserum	6·7	0·7	21/35	2·5	**6·9**	2/35
Normal mouse serum	6·5	0·9	20/35	9·2	0·2	32/35
Control (no mouse serum)	7·4	—	24/35	9·4	—	34/35

Equal volumes of heat inactivated serum and ectromelia suspension incubated together at 20°–25° for 30–40 min. and 0·2 ml. inoculated i.p. into each mouse.

E. coccoides-MHV1 mixture was neutralized no better by serum against ectromelia than by normal mouse serum and ectromelia was not neutralized appreciably better by serum against MHV1 than by normal mouse serum. The serum against ectromelia neutralized the homologous virus to high titre but it is doubtful whether the amount of homologous neutralization by the serum against MHV1 is statistically significant although its magnitude conforms to previous experience. As already stated, experiments showed that the sparing effect of infection with MHV1 is transient. Thus, it was found that when a group of mice was inoculated with the usual dose of MHV1 intraperitoneally 9 days before infection with about 1 LD_{50} of ectromelia the mortality was as high as in the control mice (see Table III). If the reduced pathogenicity of ectromelia in mice pretreated with MHV1

TABLE III.—*Survival Rates of Mice Inoculated with MHV1 1 hour and 9 days before Ectromelia Inoculation*

Pre-treatment of mice	Survivors Mice in group	Percentage survivors
MHV1 1 hr. before ectromelia	19/20	95
MHV1 9 days before ectromelia	11/20	55
Control (no pretreatment)	11/20	55
	9/22	41

Dose of ectromelia 1 LD_{50} intraperitoneally (0·2 ml.).
The survivor ratio for the group of mice treated with MHV1 1 hr. before ectromelia infection is significantly greater than that for the group of mice so treated 9 days before ectromelia infection ($\chi^2 = 6·52$, $n = 1$, $p = 0·01$).

were due to the production of antibody which neutralizes ectromelia, treatment with MHV1 9 days before infection with ectromelia would be expected to reduce the pathogenicity of ectromelia more than, not less than, when given a day or

an hour before ectromelia. The administration of MHV1 intraperitoneally 24 hr. or more after the inoculation of about 300 LD_{50} ectromelia given by the same route had no influence on its pathogenic action. The result of such an experiment is shown in Table IV.

TABLE IV.—*Null Effect of MHV1 Inoculation after Ectromelia Inoculation*

Time of MVH1 inoculation (time of ectromelia inoculation = 0)	Inoculated with MHV1: Mice which died / Mice in group	Inoculated with MHV1: Average survival time of mice which died (days)	Not inoculated with MHV1: Mice which died / Mice in group	Not inoculated with MHV1: Average survival time of mice which died (days)
− 2 hr.	7/10	9·3	10/10	6·5
+ 1 day	10/10	6·2	10/10	6·1
+ 2 days	9/9	6·4	10/10	6·5
+ 3 days	10/10	6·3	10/10	6·3
+ 4 days	10/10	7·0	10/10	6·0
+ 5 days	10/10	6·6	10/10	6·0

Dose of ectromelia : 300 LD_{50} i.p. (0·2 ml.).
Dose of MVH1 : 10^3 ID_{50} i.p. (0·2 ml.).

In experiments described so far, MHV1 and ectromelia were inoculated intraperitoneally. Although other routes of inoculation were not extensively investigated, in one experiment 2 groups (B and E) of 10 mice were inoculated intravenously (i.v.) and 2 similar groups (A and D) i.p. with the usual dose of MHV1. After 24 hr. mice of groups A and B together with a normal group (C) were inoculated i.v. with 10^3 LD_{50}s of ectromelia, and mice of groups D and E together with a group of normal mice (F) were inoculated with the same dose of ectromelia given intraperitoneally. The results of this experiment are shown in Table V.

TABLE V.—*MHV1 and Ectromelia Inoculated Intravenously and Intraperitoneally*

Group designation	Route of inoculation: Ectromelia	Route of inoculation: MHV1	Proportion of survivors 10 days after ectromelia inoculation	Probability that difference is due to chance: χ^2	Probability that difference is due to chance: p
A	i.v.	i.p.	14/20	3·0	<0·1
B	,,	i.v.	15/20	4·2	<0·05
C	,,	None	9/23	—	—
D	i.p.	i.p.	14/20	12·2	<0·001
E	,,	i.v.	9/20	4·0	<0·05
F	,,	None	5/23	—	—

MHV1 10^3 LD_{50} 24 hr. before 10^3 LD_{50} ectromelia.
i.v. = intravenous.
i.p. = intraperitoneal.

It will be seen that pretreatment with MHV1, whether given i.p. or i.v., increased the survival rate of mice inoculated i.v. or i.p. with 10^3 LD_{50} of ectromelia. The figures suggest that the interference effect appears somewhat greater when the route of inoculation of both viruses is the same. In conformity with other experience, ectromelia seems somewhat less pathogenic by the intravenous route than by the intraperitoneal route.

The Sparing Effect of Serum from Mice Infected with MHV1 upon Infection with Ectromelia

Adult VS mice were inoculated i.p. with MHV1 in the usual dose and bled out 3, 4 and 7 days afterwards. When a serum from adult mice inoculated with MHV1 4 days previously was titrated i.p. in decimal dilutions from 10^{-1} to 10^{-4} in groups of 5 mice pretreated with *E. coccoides*, one mouse of the group which received virus diluted 10^{-1} died of hepatitis, while the rest showed no lesions of hepatitis when sacrificed one week afterwards. The sera were heated at 56° for 30 min. to inactivate the small amount of virus present (Gledhill *et al.*, 1955) and the criterion that this had been accomplished was the absence of fatal hepatitis or macroscopically evident hepatitis at autopsy when 0·2 ml. of serum was injected i.p. into *E. coccoides* pretreated mice. Comparable normal mice were bled out and their serum heated in the same way. Mice of strain VS in groups of 20 or more were inoculated i.p. with 0·4 ml. of serum from MHV1 infected mice and normal serum. After 3 hr. the mice together with a group of 20 normal mice were inoculated by the same route with about 10 LD_{50} of ectromelia virus. Deaths were recorded daily. Mice inoculated with sera from mice bled out 7 days after infection with MHV1 did not differ from mice inoculated with normal mouse sera in their response to ectromelia but sera from mice bled out 3 and 4 days after MHV1 infection prolonged the survival time. In the first experiments with 3 and 4 day serum, the survival rate in each group on the first day on which the mortality in the controls exceeded 50 per cent is presented in Table VI. It will be seen that at this stage of both experiments,

TABLE VI.—*Increased Survival Time of Ectromelia Infected Mice Pre-inoculated with Serum from Mice Infected with MHV1*

Pre-treated with serum from mice	Mice surviving 8 days after virus inoculation		Probability that difference is due to chance	
	Proportion	Per cent	χ^2	p
Bled 3 days after infection with MHV1	12/20	60 } 40	5·10	≒0·02
Not infected with MHV1	4/20	20 }		
Control (no serum)	8/19	42		
Bled 4 days after infection with MHV1	17/39	44 } 34	9·38	<0·01
Not infected with MHV1	4/39	10 }		
Controls (no serum)	3/20	15		

All sera heated to 56° for 30 min. to inactivate MHV1.
Serum : 0·4 ml. i.p. 3 hr. before virus.
Ectromelia : about 10 LD_{50} i.p. (0·2 ml.).

the survival rates were higher in the groups of mice which had received serum from mice infected with MHV1 than in the groups which had received normal mouse serum and in the controls which had received no mouse serum. Similar experiments with serum from mice bled 3 and 4 days after infection with MHV1 were repeated and these experiments included groups of mice which were inoculated with serum-virus mixtures as well as groups of mice inoculated with serum 3 hr. before virus, as heretofore. The results are shown in Table VII. It will be seen that more mice again survived in the groups which received serum from

TABLE VII.—*Increased Survival Time of Ectromelia Infected Mice Inoculated with Serum from Mice Infected with MHV1 : (a) Serum Pre-inoculated, and (b) Serum Incubated with Virus*

	Treated with serum from mice	Serum–virus sequence: Serum before virus (+ or −)*	Serum–virus sequence: Serum mixed with virus (+ or −)†	Mice surviving 8 days after virus inoculation: Proportion	Mice surviving 8 days after virus inoculation: Per cent		Probability that difference is due to chance: χ^2	Probability that difference is due to chance: p
Exp. A	Bled 3 days after infection with MHV1	+	−	11/20	55	20	0·91	÷0·3
	Not infected with MHV1	+	−	7/20	35			
	Bled 3 days after infection with MHV1	−	+	13/20	65	50	8·4	<0·01
	Not infected with MHV1	−	+	3/20	15			
	Controls (no serum)	−	−	6/20	30			
Exp. B	Bled 4 days after infection with MHV1	+	−	12/19	63	38	4·3	<0·05
	Not infected with MHV1	+	−	5/20	25			
	Bled 4 days after infection with MHV1	−	+	11/20	55	29	2·3	÷0·20
	Not infected with MHV1	−	+	5/19	26			
	Controls (no serum)	−	−	8/19	42			

All sera heated to 56° for ½ hr. to inactivate MHV1.
* Serum : 0·4 ml. i.p. 3 hr. before virus. Ectromelia : about 10 LD_{50} i.p. (0·2 ml.).
† Serum (0·4 ml.) mixed with about 10 LD_{50} extromelia (0·1 ml.), incubated for 20 min. at 37° in Experiment A, at 20°–25° in Experiment B and injected i.p. (0·5 ml.).

mice infected with MHV1 than in those which received normal mouse serum and in the controls. The survivor ratio for both groups shown in Table VII which received serum from MHV1-infected mice mixed with ectromelia is

$$\frac{24}{40} \text{ (60 per cent)}$$

and the survivor ratio for both groups which received these sera 3 hr. before ectromelia is

$$\frac{23}{39} \text{ (59 per cent)}$$

If the sparing effects recorded were due to direct inactivation of ectromelia by the sera the survivor ratio for mice given serum-virus mixtures might be expected to be much higher than that for mice given serum 3 hr. before virus. The total survivor ratios from the figures shown in Tables VI and VII are as follows :

	Survivors: Ratio	Survivors: Per cent	Difference per cent
Mice which received inactivated MHV1 serum	76/138	55	35
Mice which received inactivated normal serum	28/138	20	
Controls (no serum)	25/78	32	12

The survivor ratio of mice which received MHV1 serum is significantly higher than that of mice which received normal serum ($p < 0{\cdot}001$) and that of the

control mice which received no serum ($p < 0{\cdot}01$). It will be observed that the survivor ratio of the mice which received normal serum is depressed 12 per cent below that of the control mice but the trend of subsequent experiments (see below) did not confirm the significance of this difference.

An experiment was undertaken to determine whether serum from mice infected with MHV1 would retain its sparing action after filtration through a Gradocol membrane of average pore diameter (APD) sufficiently small to prevent the passage of infectious MHV1 particles. Adult mice were bled 3 days after the usual infection with MHV1 and half the serum obtained and also serum from comparable normal mice were each filtered through preparatory Gradocol membranes. The operation was completed by filtration through membranes of APD 130 mμ and the filtrate of the MHV1 serum, tested in *E. coccoides* pretreated mice, as detailed above, did not reveal the presence of MHV1. The other half of the infected serum and normal serum were heated as usual and the 4 sera were inoculated into groups of mice which were infected with ectromelia 3 hr. afterwards, as in previous experiments. It will be seen from the results shown in Table VIII that there was no significant difference between the filtered

TABLE VIII.—*Comparison of Serum from Mice Infected with MHV1 in which Virus was (a) Removed by Filtration and (b) Inactivated by Heat*

Source of serum	Treatment of serum	Mice surviving 8 days after virus inoculation: Proportion	Per cent	Difference per cent	Number of mice which finally survived	Mean survival time of mice which died (days)
MHV1 infected mice	Filtered	14/20	70	35	2	$9{\cdot}3 \pm 1{\cdot}2$
Normal ,,	,,	7/20	35		2	$8{\cdot}1 \pm 1{\cdot}5$
MHV1 infected ,,	Heated	11/20	55	35	2	$9{\cdot}0 \pm 1{\cdot}4$
Normal ,,	,,	4/20	20		1	$7{\cdot}9 \pm 1{\cdot}2$
Control (no serum)	—	5/20	25	—	0	$8{\cdot}2 \pm 1{\cdot}1$

MHV1 serum from adult mice bled 3 days after i.p. inoculation of MHV1.

Serum, filtered through Gradocol membrane of 130 mμ or heated at 56° for 30 min., inoculated i.p. 3 hr. before 10 LD_{50} ectromelia i.p.

Probability that mean survival times differ by chance between mice which received :

(*a*) Filtered MHV1 serum (9·3 days) and heated MHV1 serum (9·0 days) : $t = 0{\cdot}7625$, $n = 35$, $0{\cdot}5 > p > 0{\cdot}4$.

(*b*) Filtered MHV1 serum (9·3 days) and filtered normal serum (8·1 days) : $t = 2{\cdot}571$, $n = 34$, $p < 0{\cdot}02$.

(*c*) Heated MHV1 serum (9·0 days) and heated normal serum (7·9 days) : $t = 2{\cdot}489$, $n = 35$, $p < 0{\cdot}02$.

and heated sera and that both the MHV1 sera increased significantly the survival time in comparison with both the normal sera. In a confirmatory experiment with another serum bled from adult mice 3 days after infection with MHV1 and filtered through a Gradocol membrane of 130 mμ, the following result was obtained :

Type of material	Mice surviving 7 days after virus inoculation: Proportion	Per cent	Difference per cent	Probability that difference is due to chance: χ^2	p
Filtrate of infected serum	16/18	89	39	4·95	$<0{\cdot}05$
Filtrate of normal serum	10/20	50			
Controls (no serum)	9/20	45			

As before, no MHV1 was demonstrable in the filtrate of serum from the infected mice. The result was similar to that of the previous experiment and it was concluded that infected serum from which infectious virus had been removed by filtration increases the survival time to about the same extent as infected serum in which infectious virus has been inactivated by heat.

DISCUSSION

The evidence brought forward to show that mouse hepatitis virus does not evoke the production of a detectable amount of antibody against ectromelia seems to exclude the hypothesis that neutralizing antibody is responsible for the sparing effect on ectromelia infection. When phenomena of this type are not explicable by antibody production, interference is the only available term to apply to the protective effect of a virus, whether active or inactivated, against the pathogenic action of another virus. Interference in this sense probably includes effects referable to different mechanisms and with further knowledge, the term interference may become restricted to a particular kind of mechanism. At present we must class as interference the effect of prior infection with MHV1 upon the pathogenicity of ectromelia.

The sparing effect upon ectromelia of serum from mice 3 and 4 days after inoculation of MHV1 and its disappearance from serum 7 days after inoculation of the virus suggest that this property of serum during the limited period might account for the transient interfering action of the virus. The fact that inoculation of incubated serum-virus mixtures did not result in a greater sparing effect than inoculation of sera 3 hr. before virus shows that the sera do not inactivate ectromelia directly but act by raising the resistance of mice towards its pathogenic effects. It might be argued that the sparing effect is due to MHV1 in such sera. However, it is known that MHV1-containing suspensions are inactivated by heating at 56° for 30 min. and, moreover, attempts to demonstrate active virus in heated sera used in the experiments described in this paper were unsuccessful. Since removal of infective virus by filtration can replace inactivation by heat without adverse action upon the sparing effect it is unlikely that interference with ectromelia is due to heat inactivated MHV1. Moreover, it is well known that very large amounts of inactivated virus are required to produce demonstrable interference in animals and serum from mice bled 4 days after inoculation with MHV1 and tested before heat treatment or filtration contained barely demonstrable amounts of virus. The simplest explanation is that infection with MHV1 has resulted in the presence in the blood of a substance which has this sparing action.

The resistance of mice to intraperitoneal infection with ectromelia appears to be greater when MHV1 is inoculated by the same route than when it is inoculated intravenously (see Table V), and it is possible that MHV1 infection in the peritoneal cavity plays an important role in the production of interference. Following i.p. inoculation of MHV1 into weanling mice, generalised changes are found in the mesothelial lining; many of the cells become enlarged, multinucleated giant cells appear and polymorphs and mononuclears accumulate in the mesentery and omentum. These cytopathological effects reach a maximum within 48 hr., after which the abnormal cells undergo rapid degeneration. The production of the sparing substance by mesothelial and inflammatory cells

altered in this way by the virus might account for the presence in serum of the sparing substance 3 days after inoculation of MHV1, especially since materials arising in the peritoneal cavity are known to pass quickly into the blood stream ; further experiments may show the substances to be already present in the serum of mice 2 days, or even one day, after virus inoculation. The liver damage which becomes obvious microscopically on the 3rd and 4th days is self-limiting and rarely progresses beyond the 7th day and thus appears to play a lesser part in the production of interference since sparing action is no longer demonstrable in serum from mice bled on the 7th day and the pathogenicity of ectromelia cannot be altered by giving MHV1 9 days previously. The hypothesis suggested above is based upon cyto- and histopathological information supplied by Dr. Janet S. F. Niven.

Since there seems to be a relationship between protective action and the occurrence of cytopathological changes, consideration must be given to the possibility that agents other than viruses may stimulate the production of sparing substances. Evidence for this view has already been recorded. As noted earlier, the serum of mice bled 2 hr. after the inoculation of bacterial endotoxin contains a substance which reduces the pathogenicity of ectromelia. Inactivated viruses may elicit an interfering effect somewhat differently. Isaacs and Lindenmann (1957) have shown that cells of isolated pieces of chick chorio-allantoic membrane react to appropriate treatment with inactivated influenza virus by liberation of a substance which they have designated interferon and which raises the resistance of susceptible cells to infection with active influenza and other viruses (Isaacs, Lindenmann and Valentine, 1957). It would therefore be of great interest to discover what biological and chemical relationships exist between these 3 substances, that is, interferon, the substance in the blood of mice inoculated with endotoxin and the substance in the blood of mice inoculated with MHV1. No attempts to define the nature of the latter substance have yet been undertaken, nor is it yet known whether the technique used is the most effective. For example, the time after infection with MHV1 at which the substance reaches its highest concentration in the blood is still unknown and the optimum interval between the injection of serum containing it and the injection of ectromelia has not yet been accurately determined. Furthermore, it is of general importance to determine whether the serum is active against other viruses both in animals and other systems capable of growing viruses, such as fertile eggs and tissue cultures. The employment of such techniques should give some indication of the site and nature of its biological activity.

SUMMARY

When weanling mice were inoculated intraperitoneally with mouse hepatitis virus (MHV1) followed by ectromelia 24 hr. afterwards, the lethal effect of the latter virus was much reduced. No evidence of cross neutralization between ectromelia and MHV1 immune sera was obtained. When MHV1 was inoculated 9 days before ectromelia or 24 hr. after ectromelia the pathogenicity of ectromelia was not reduced. The lowered pathogenicity of ectromelia for mice infected with MHV1 is classed as interference.

Serum from adult mice bled 3 or 4 days after infection with MHV1, in which infectious virus particles had been inactivated by heat or removed by filtration,

increased the survival time of mice infected with about 10 LD_{50} of ectromelia. The sparing effect is attributed to a substance which appears to raise the resistance of mice rather than to inactivate ectromelia. The production of this substance during at least 2 days by mice infected with MHV1 may explain the interference of MHV1 with ectromelia.

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SURVEY OF PAPERS

VANĚČEK, RAŠHOVA, JELÍNEK, RAŠKA and MATÉJOVSKÁ inoculated mice twice weekly with phenol. After varying numbers of doses the LD_{50} of a standard lyophilized *Sh. shigae* toxin was determined. They found that pretreatment with Phenol produced an increased resistance to the toxin. Phenol pretreatment had no effect on the toxicity of arsenic trioxide in mice (p. 203).

ASHERSON tested the sera of patients with systemic lupus erythematosus and other conditions for complement fixing antibodies with nuclear and cytoplasmic antigens prepared from human and rat liver and calf thymus; 4 sera reacting with nuclear antigens came from patients with positive LE cell tests. But sera from 9 patients with LE cells failed to react with nuclear antigens (p. 209).

HURLEY and KATHRYN HAM administered systemically the aminonitriles AAN and BAPN to rats during the development of carrageen granulomata and following the subcutaneous implantation of polyvinyl sponges. The development of carrageen granulomata was associated with blockage of collagen maturation between the 10th and 15th day. The changes in the granulation tissue induced by the implanted polyvinyl sponges were reversible on cessation of the drugs. The histological features are similar to those previously described with turpentine induced abscesses (p. 216).

ARMSTRONG and FULTON describe the changes in the pelvic viscera and livers of cotton rats following the intraperitoneal injection of *Toxoplasma gondii*. It was found that male rats developed urethral obstruction with urinary retention due to parasitic invasion and acute inflammatory changes in the pelvic organs. The livers showed a peri-portal fatty infiltration in the absence of parasitic invasion. The authors find that the identification of *T. gondii* in tissues is facilitated by the acridine orange fluorescence technique (p. 225).

WEINBREN and FITSCHEN performed partial hypophysectomies in rats to investigate whether regeneration subsequently followed. No evidence of regeneration was found in the pituitary remnants up to 9 weeks after the partial resections. These partially hypophysectomized rats were not distinguishable from the control animals on clinical grounds, nor on the basis of body or adrenal weights. The induction of goitres by methyl-thiuracil provided a means of differentiating the 2 groups by comparing the increase in thyroid epithelial cell height (p. 232).

HESS and PEARSE have observed changes in mitochondrial activity in the maculae densae of rats rendered hypertensive by unilateral clamping of a renal artery. Histochemical methods have shown that these changes are substantially confined to the ischaemic kidney and may be related to changes in the level and distribution of glucose-6-phosphate and 6-phosphogluconate dehydrogenases (p. 243).

DU RUISSEAU and MORI have measured electrolyte concentrations in the tissues of rats with heart lesions experimentally induced by the combined administration of sodium dihydrogen phosphate and 2α-methyl-9α-chlorocortisol acetate. Although changes in sodium, potassium and calcium chloride and phosphate were observed in several tissues the most consistent finding in association with cardiac lesions was a marked fall in the magnesium content of heart muscle (p. 250).

CURTAIN found that rabbits less than 56 days old, immunologically tolerant to a Bence-Jones protein, could make antibodies only to those antigenic determinants (recognised by

the Ouchterlony method) of myeloma globulin from the same patient, which the Bence-Jones protein did not have. Then he showed that tolerance to pooled human plasma prevented production of any antibodies to myeloma globulin. Later he gave massive daily doses of normal human plasma to adult rabbits, in order to produce antibodies to minor but not main components of plasma. Using coated tanned sheep cells, he found that the sera of these rabbits reacted more strongly with myeloma globulins than with normal γ-globulin. An explanatory hypothesis is offered (p. 255).

HEPPLESTON and KEYSER have measured changes in the serum protein-bound carbohydrate levels in rats bearing the Walker 256 carcinoma. Significant rises in hexose-binding by total serum protein, by a_2-globulin, by mucoprotein and non-mucoprotein could not be correlated with the amount of viable or necrotic tissue present (p. 263).

PORTER irradiated rabbits in divided doses to a total of 1600r (LD_{100} 30 days). The rabbits were given intravenously, within 3 hr. of the last X-ray dose, adult marrow, neonatal liver, 27 or 20 day foetal liver from donor rabbits, or nothing (controls). All controls died within 2 weeks, whereas the various haemopoietic transplants were equally effective in preventing early death. However, 20-day foetal liver was more successful than other materials in preventing death during the second month from " secondary disease " (p. 273).

HOWARD, BIOZZI, HALPERN, STIFFEL and MOUTON injected Swiss albino mice intravenously with *Myco. tuberculosis* (BCG). Two weeks later colloidal carbon, killed *Salm. typhi*, lipopolysaccharide from *Bact. coli* (with or without ^{32}P), or living *Salm. enteritidis* (with or without ^{51}Cr) were injected intravenously. These workers found that carbon, endotoxin and live bacteria were cleared from the blood more rapidly than in controls, and *Salm. enteritidis* killed more slowly, but susceptibility to the endotoxin of *Salm. typhi* was increased (p. 281).

GLEDHILL inoculated weanling mice with mouse hepatitis virus (MHV 1) or with serum from adult mice, inoculated 3–4 days earlier with MHV 1, which had been cleared of virus by heating or filtration. The former procedure protected mice infected 24 hr. later with ectromelia, but not those infected 9 days later. The serum was protective when mixed with or administered 3 hr. before the ectromelia. It was ineffective if withdrawn from its donors 7 instead of 4 days after their infection with MHV 1. No cross-neutralization could be demonstrated between MHV 1 and ectromelia, and the author attributes his findings to some form of interference (p. 291).